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Using various lines of evidence to identify *Chironomus* species (Diptera: Chironomidae) in eastern Canadian lakes

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Abstract

Chironomus Meigen (Diptera, Chironomidae) larvae are usually the largest sediment-burrowing chironomids, and as such often constitute a major part of the freshwater infaunal biomass. However, use of this genus in ecological, environmental and paleoecological studies is hampered by the fact that *Chironomus* larvae are difficult to identify to species because the larvae of many species are morphologically similar. We used a combination of morphological, cytological and genetic techniques to distinguish *Chironomus* larvae collected from 31 water bodies located in eastern Canada, producing 17 distinguishable groupings. These groups of larvae were ultimately identified as belonging to 14 known species (*C. anthraci-*

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nus, *C. bifurcatus*, *C. cucini*, *C. decorus*-group sp. 2, *C. dilutus*, *C. entis*, *C. frommeri*, *C. harpi*, *C. maturus*, *C*. nr. *atroviridis* (sp. 2i), *C. ochreatus*, *C. plumosus*, *C. staegeri* and *C. 'tigris'*) and three other species that remain unidentified (*C*. sp. NAI-III). No single approach served to delimit and identify larvae of all 17 *Chironomus* species that we collected. Although we expected that morphological criteria alone would be insufficient, our results suggest that DNA barcoding, using either the mitochondrial *cox1* or the nuclear *gb2β* gene, was also inadequate for separating some *Chironomus* species. Thus we suggest that multiple approaches will often be needed to correctly identify *Chironomus* larvae to species.

Key words: *Chironomus*, morphology, cytology, DNA barcoding, *cox1*, *gb2β*, Canada

Introduction

The insect genus *Chironomus* Meigen (Diptera, Chironomidae) is found in fresh waters on all continents except Antarctica. It includes several hundred species, now classified into three subgenera (*Chaetolabis*, *Chironomus*, *Lobochironomus*) (the subgenus *Camptochironomus* is no longer recognized—see Sæther (2012)), as well as other species that are yet to be described (Ryser *et al.* 1985; Ashe & Cranston 1990; Martin 2013). In lakes from the tropics (Hare & Carter 1986), to the temperate (Jónasson 1972), to the Arctic (Butler 1982), *Chironomus* larvae are usually the largest sediment-burrowing chironomid and often represent a major part of the infaunal biomass. Thus *Chironomus* larvae can be an important source of food for fish and are widely used in ecological (Jónasson 1972), environmental (Martin *et al.* 2008) and paleoecological (Brooks *et al.* 2007) studies of fresh waters. If we are to understand their roles in aquatic ecosystems, it is important to be able to correctly identify *Chironomus* species.

The identification of *Chironomus* larvae to species can be problematic because there are few conspicuous morphological differences among many *Chironomus* species (Lindeberg & Wiederholm 1979). As a result, larvae are often referred to simply as *Chironomus* spp. (Nyman *et al.* 2005) or at best are grouped into types according to the presence and form of their abdominal tubules (Shobanov *et al.* 1996) or the shape of their mouth parts (Brooks *et al.* 2007). Such groupings can limit the use of *Chironomus* larvae in ecological, environmental and paleoecological studies because behavioural and ecological differences among species are often important. For example, cadmium concentrations in sympatric *Chironomus* species can vary by an order of magnitude because of differences in their feeding habits and consequent contaminant exposure (Martin *et al.* 2008; Proulx & Hare 2008, 2013). Pooling such species would clearly limit their use as contaminant biomonitors. If we cannot correctly identify *Chironomus* larvae to species, then it is difficult to use them to infer environmental impacts.

In early studies, features of the head capsule and abdominal tubules were used to identify *Chironomus* larvae to species (Johannsen 1937). Subsequently, *Chironomus* species were also separated on the basis of the structure of polytene chromosomes located in their salivary glands (Keyl 1962; Martin 1979; Wülker *et al.* 1989). In the last decade or so, genetic techniques have been used to supplement these earlier taxonomic methods. For example, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach has been used to produce species-specific DNA profiles that can differentiate among *Chironomus* species (Carew *et al.* 2003; Sharley *et al.* 2004). This technique involves first amplifying specific genes or regions with PCR, and then digesting the resulting PCR amplicons with restriction endonucleases. Restriction endonucleases cut PCR amplicons differentially based on nucleotide differences in their DNA sequence, thereby generating a species-specific RFLP or DNA profile. The DNA profiles are visualised by gel electrophoresis as DNA fragments of different lengths. Although this method is inexpensive and useful for screening large numbers of individuals, it only examines a subset of the variation present in PCR amplicons (Pfrender *et al.* 2010).

Another genetic technique used to separate and identify species is DNA sequencing (also known as Sanger sequencing) of PCR amplicons. This technique, referred to as DNA barcoding when used for identifying species, is more exact than PCR-RFLP as it detects all nucleotide differences. The standard gene used for DNA barcoding is the 3' end of the mitochondrial *cytochrome oxidase subunit I* (*cox1*; Hebert *et al.* 2003). Advantages of using the *cox1* gene are that universal primers are able to amplify this gene from many animal groups (Folmer *et al.* 1994) and sequence variations in *cox1* can be used to discriminate among many closely-related species (Hebert *et al.* 2004a). In insects, DNA barcoding using the *cox1* gene has been used to identify species from a range of groups including the Collembola (Hogg & Hebert 2004), the Ephemeroptera (Ball *et al.* 2005; Elderkin *et al.* 2012), the Coleoptera (Davis *et al.* 2011) and the Chironomidae (Carew *et al.* 2007; Ekrem *et al.* 2007; Pfenninger *et al.* 2007; Sinclair & Gresens 2008; Ekrem *et al.* 2010; Carew *et al.* 2011; Stur & Ekrem 2011). Although *cox1* sequences can

be used to separate the majority of species, its mitochondrial origin is problematic for *Chironomus* because some species are known to hybridize (Martin 2011). Therefore, including sequence data from additional nuclear markers whose mode of inheritance differs from mitochondrial genes is required (Guryev *et al.* 2001; Martin *et al.* 2002; Martin 2011). To this end, the nuclear gene *globin 2β* (*gb2β*) has been used in several studies on *Chironomus* species (Kao *et al.* 1994; Hankeln *et al.* 1997; Guryev *et al.* 2001; Guryev & Blinov 2002; Martin *et al.* 2002).

We applied morphological, cytological and genetic techniques to identify *Chironomus* larvae collected in 31 water bodies in eastern Canada to determine what combination of techniques would allow us to accurately identify the *Chironomus* species. To date, very few studies have used multiple techniques to discriminate among *Chironomus* species. We anticipate that the results of our study will be useful to those wishing to identify North American *Chironomus* species and will provide useful tools to those wishing to identify *Chironomus* species on other continents. The ability to accurately identify *Chironomus* species should facilitate future ecological and environmental studies in this and other geographical zones.

Methods

Collection and dissection of larval *Chironomus.* We collected fourth-instar *Chironomus* larvae from 31 water bodies (Table 1) located in the provinces of Quebec (near Quebec City, Rouyn-Noranda and Trois-Rivières) and Ontario (near Sudbury), Canada. The collection period extended from ice-off in late spring (May) to early summer (June) in various years from 2006 to 2011 (Table 1). Exceptionally, in Lake Bédard, *Chironomus* were collected at the end of the summer (September). Sediments were collected using an Ekman grab and sieved through a net to eliminate fine sediment and retain *Chironomus* larvae. Larvae were preserved in 94% ethanol.

Depth and water chemistry were measured at each collecting site (Table 1). Water samples were filtered *in situ* using diffusion samplers (Ponton & Hare 2009). Dissolved organic carbon (DOC) was measured by combustion and transformation into CO_2 (TOC-VCPH, Shimadzu, Columbia, MD, USA) and magnesium (Mg) and calcium (Ca) concentrations were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Vista AX CCD, Varian, Mississauga, Ontario, Canada). Quality assurance of water chemistry measurements was assured through the use of blanks and appropriate standard reference materials. At the time of sampling, the water columns of all lakes were well mixed and oxygenated.

Head capsule and terminal abdominal segments with attached tubules were separated and kept for morphological studies, whereas the rest of the body was retained for genetic analyses. In addition, three individuals of each species (as determined by genetic analyses and morphology) were chosen at random for examination of their polytene chromosomes. For this purpose, the thoracic segments containing the salivary glands were preserved in a 3:1 mixture of 94% ethanol to glacial acetic acid (not all specimens showed chromosomal patterns of sufficient quality for species identification). Exceptionally, 41 larvae of *C. entis* and C. *plumosus* were examined cytologically to validate genetic results for these species.

Genetic analyses. Polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP) analysis was performed on *Chironomus* larvae collected in 2006 and 2007. Specimens of each PCR-RFLP profile were subsequently sequenced for further DNA analysis. Specimens collected in subsequent years were sequenced directly.

DNA extraction. DNA was extracted from larvae using the modified Chelex method (Carew *et al.* 2003). Briefly, the larval body (minus the head and terminal segments) was dried using a paper towel and placed in a 0.5 mL plastic microcentrifuge tube. Individuals with large amounts of sediment in their gut were avoided as this can inhibit the PCR (Carew *et al.* 2003). Tubes were immersed in liquid nitrogen and the contents crushed into a powder using a pestle, 400µL of suspended 5% Chelex-100 resin (BioRad) was added and samples were incubated at 90 °C for 30 min. Extracts were stored at -20 °C until required for the PCR procedure.

Polymerase chain reactions. PCR amplification of portions of the *cox1* and the *gb2β* genes was carried out in a 40 µL reaction mixture containing: 1x PCR pH 8.8 buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100; New England Biolabs (NEB)), 200 µM each of deoxynucleotide triphosphate (dNTPs), 0.4 mg/mL of bovine serum albumin (BSA), 0.5 µM of forward and reverse primers (see Table 2), 1 unit of *Taq* DNA polymerase (NEB), and 5 µL of Chelex DNA extraction supernatant taken from just above the resin after centrifugation at 15,000 relative centrifugal force for 2 min (Carew *et al.* 2003). All *gb2β* gene primers tested

TABLE 1. Location, year and depth of collection, as well as water chemistry and trophic status of the water bodies studied.

Water body	Code	Year	Depth (m)	Maximum depth (m)	Location	Water chemistry				Trophic status[b]
						pH	Ca (µM)	Mg (µM)	DOC (mg/L)	
QUEBEC CITY (QC)										
Lake Bédard	BE	2009	4–6	10	47°16'N, 71°07'W	5.7–8.3	35–69	11–17	3.6–5.3	mesotrophic[c]
Lake Saint Augustin	AU	2010	3,5		46°45'N, 71°24'W					eutrophic (2003)[d]
St. Charles River	SC	2010	1–3	3	46°49'N, 71°13'W					
Lake St. Joseph	SJ	2006	6–24	37	46°53'N, 71°38'W	7.1				oligo–mesotrophic[d]
ROUYN-NORANDA (QC)										
Lake Adéline	AD	2007			48°12'N, 79°10'W					
Lake Arnoux	AR	2010	1.5–4.5	4.5	48°15'N, 79°20'W	3.8–4.4	286–296	146–156	0.1–1.1	
Lake Bousquet	BO	2006	14	18	48°13'N, 78°39'W	6.9[a]				
Lake D'Alembert	DA	2006	5		48°23'N, 79°01'W					eutrophic (2009–2011)[d]
Lake Dasserat	DS	2006, 2010	3–5	17	48°17'N, 79°25'W	7.5–7.6	205–223	97	5.9–6.3	
Lake Dufault	DF	2006	4	19	48°17'N, 79°00'W	7.7	392	110	3.8	oligo-mesotrophic (2010)[d]
Lake Duprat	DP	2006, 2007, 2010	5–7	7.5	48°20'N, 79°07'W	6.8–7.6	140–178	45–57	2.9–6.6	
Lake Fortune	FO	2006	5–6		48°11'N, 79°19'W	7.6				oligo-mesotrophic (2008)[d]
Lake Kinojévis	KI	2006	7–8		48°08'N, 78°54'W	7	363	109	5.6	
Lake Marlon	MN	2006, 2007, 2009, 2010	1–2	2	48°16'N, 79°04'W	7.1–7.7	160–168	58–61	8.4–7.8	meso-eutrophic[d]
Lake Opasatica	OP	2006, 2007, 2009	2–9	60	48°10'N, 79°20'W	7.4–8.0	213–216	107–115	6.7–7.4	mesotrophic (2008)[d]
Lake Osisko	OS	2006, 2009, 2010	5.5, 6.5	6.5	48°15'N, 79°00'W	7.8–8.5	690	183	2.3	
Lake Pelletier	PE	2010	5		48°13'N, 79°03'W	8.3	826	258	3.7	meso-eutrophic[d]
Lake Rouyn	RO	2010	3.5–4		48°15'N, 78°57'W	8	2060	289	3.5	meso-eutrophic[d]
Lake Vaudray	VA	2010	35	35	48°04'N, 78°41'W	7.1	79	36	8.7	oligotrophic (2011)[d]
TROIS-RIVIERES (QC)										
unnamed pond	PO	2007	1	1	46°13'N, 72°39'W					

... ... *continued on the next page*

TABLE 1. (Continued)

Water body	Code	Year	Depth (m)	Maximum depth (m)	Location	Water chemistry				Trophic status[b]
						pH	Ca (µM)	Mg (µM)	DOC (mg/L)	
SUDBURY (ON)										
Kasten (Bibby) Lake	KA	2007	7.5	8	46°22'N, 80°58'W	6.8	69	45	4.4	oligotrophic (2008)[e]
Clearwater Lake	CL	2007	19	19	46°22'N, 80°03'W	6.2	109	43	2.3	oligotrophic[e]
Crooked Lake	CR	2007	5-6	8	46°25'N, 81°02'W	6.7	71	48	3.6	oligotrophic[e]
Hannah Lake	HA	2007, 2010	7–7.5	7.5	46°27'N, 81°02'W	7.4–7.9	258–265	147–151	3.5–3.7	oligotrophic[e]
Kelly Lake	KE	2010, 2011	1.5–5	17	46°27'N, 81°04'W	7.5, 8.4, resp.	4596, 3683, resp.	1267, 614, resp.	7.0, 5.1, resp.	eutrophic (2008)[e]
McFarlane Lake	MC	2007	10	18	46°25'N, 80°57'W	7.8	430	227	4.2	oligo-mesotrophic[e]
Pine Lake	PI	2010	4–6	6	46°23'N, 81°01'W	5.7	25	15	1.7	
Raft Lake	RA	2010	10	14	46°25'N, 80°57'W	7.3	78	45	2.2	oligotrophic[e]
Ramsey Lake	RM	2007	12	18	46°28'N, 80°57'W	7.1	381	193	3.1	mesotrophic[e]
Silver Lake	SI	2007, 2011	4	10	46°26'N, 81°01'W	5.9, 7.0, resp.	194, 283, resp.	117, 170, resp.	2.7, 3.4, resp.	oligotrophic[e]
Tilton Lake	TI	2007–2011	4	12	46°21'N, 81°04'W	6.6, 7.1, resp.	89, 78, resp.	40, 37, resp.	2.3, 3.2, resp.	oligotrophic[e]

[a] Fortin *et al.* (2010)

[b] Trophic status determined for the collecting years, unless mentioned otherwise in parentheses

[c] Trophic status inferred from total phosphorus and chlorophyll a (data not published, personal communication from Jean-Christian Auclair, INRS – Centre Eau Terre Environnement)

[d] Trophic status inferred from total phosphorus, chlorophyll *a* and water transparency (Ministère du Développement durable de l'Environnement et des Parcs 2012)

[e] Trophic status inferred from total phosphorus measurements (City of Greater Sudbury 2013)

in our study are listed in Table 2. The primers used to amplify the *gb2β* gene for each species are given in Table 4. For the *cox1* gene, the PCR thermal regime consisted of an initial denaturation cycle of 94 ºC for 3 min; followed by 40 cycles of denaturation at 94 ºC for 15 s, annealing at 45 ºC for 45 s, elongation at 72 ºC for 1 min; and one cycle at 72 ºC for 1 min. The PCR thermal regime for the *gb2β* gene was the same, but in some instances an annealing temperature of 50 ºC was used. All PCRs had a negative control with no DNA template added. PCR products were verified by electrophoresis on a 1.5% Tris-Acetate-EDTA agarose gel with ethidium bromide. PCR product sizes were estimated using Hyper Ladder II (Bioline).

TABLE 2. Primers used in this study.

Gene	Primer (forward (for) or reverse (rev))	Sequence (5'-3')	Reference
cox1	911 (for)	TTTCTACAAATCATAAAGATATTGG	Folmer *et al.* (1994)
	912 (rev)	TAAACTTCAGGGTGACCAAAAAATCA	
gb2β	wyk1b (for)	GAYATCCTTTACTACTYTT	Modified version of Kao *et al.* (1994) wyk1 primer
	wyk4 (rev)	GACCTTGTGTCCAGGC	Kao *et al.* (1994)
	wyk3 (rev)	GTGTTTCCATAGCTGGC	
	2β-B (for)	GATATCCTTTACTACATC	Hankeln *et al.* (1997)
	2β-A (rev)	CGATGTCAATAAATACATG	
	*2β*con for (for)	CCAGACATCATGGCTAA	
	*2β*con rev (rev)	CTTGACAACATCTTCGAC	

Polymerase chain reaction-restriction fragment length polymorphism analysis. Digest enzymes were chosen based on previous publications on chironomid identification using PCR-RFLP (Carew *et al.* 2003; Sharley *et al.* 2004; Carew *et al.* 2005; Carew *et al.* 2007). The PCR products from *cox1* were cleaved using restriction endonucleases with 4 base pairs (bp) (*Alu* I, *Rsa* I, *Taq* I) and 6 bp (*Hha* I, *Hinf* I, *Ssp* I) recognition sites. All digests were carried out as described by Carew *et al.* (2003) in a 20 µL reaction mixture containing: 10 µL of PCR product, 1x recommended buffer, 0.1 mg/mL BSA and variable units of restriction endonucleases (3 units for *Alu* I, *Rsa* I and *Ssp* I, 4 units for *Taq* I and 6 units for *Hha* I and *Hinf* I; NEB). Restriction digests were incubated at 37 ºC overnight, with the exception of *Taq* I, which was incubated at 65 ºC for 3h. Digest products were separated via electrophoresis for 2h at 100V on a 3% agarose gel stained with ethidium bromide and observed under UV light. The size of digest fragments was estimated with a 50 bp ladder (Promega). Fragment sizes below 100 bp were ignored, as they were not always clearly discernible on the agarose gels. To verify results obtained from these digests, we simulated digests of the corresponding *cox1* sequences using the New England BioLabs NEBCutter V2.0 program (http://tools.neb.com/NEBcutter2/). Simulation digests were also performed on the *cox1* sequences of larvae for which RFLP digests were not made.

DNA sequencing analysis. The *cox1* (709 bp) and *gb2β* (332–394 bp) gene products were purified and sequenced in both directions using the forward and reverse primers used for PCR amplification by Macrogen (Seoul, Korea) or by the research center at the Centre hospitalier universitaire de Québec (Quebec, Canada) on an ABI3730 XL automatic DNA sequencer (Applied Biosystems) and were aligned using BioEdit 7.1.3.0 (Hall 1999). All sequences used for DNA analyses were submitted to GenBank (KF278208-KF278447; KF278449-KF278450). Sequences from *cox1* were aligned using CLUSTAL W (Thompson *et al.* 1994). Due to the presence of introns in some species, *gb2β*-sequences were aligned manually according to Hankeln *et al.* (1997). Sequences were analyzed in MEGA 5.05 (Tamura *et al.* 2011). Primer sequences for each gene were excluded from the analysis. Since our goal was to separate *Chironomus* species based on sequence similarities, rather than to infer interspecific phylogenetic relationships, identification trees (ID-trees) based on *cox1*-sequences and *gb2β*-sequences were built using the Neighbor-Joining (NJ) (Saitou & Nei 1987) algorithm. The pairwise distances were calculated from the Kimura 2-parameter (K2P) model (Kimura 1980), which is best suited when distances are low (Nei & Kumar 2000), as in our study. Bootstrap analysis was performed with 1000 replicates. For the *cox1* identification (ID) tree, *Polypedilum aviceps*, *Drosophila affinis* and *Glyptotendipes lobiferus* sequences from GenBank were added as

outgroups. In the case of the *gb2β* ID-tree, because this gene is quite variable and because the only really conserved regions are also conserved in the *globin* genes *7A* (*gb7A*) and *9* (*gb9*) (Hankeln *et al.* 1997), *Chironomus* species sequences of the *gb7A* and *gb9* genes were also added to make sure that all sequences obtained for our specimens were of the *gb2β* gene. The *gb2β* primers did in fact amplify the *gb7A* gene from *C*. (*Chaetolabis*) nr. *atroviridis* (sp. 2i). Pairwise intraspecific and interspecific nucleotide-sequence divergences were also calculated for all sequences using the K2P model in MEGA 5.05. Since some authors have expressed reservations about using divergence thresholds to separate species (DeSalle *et al.* 2005), including those of *Chironomus* (Martin 2011), we also used specific base differences to quantify differences between some closely-related species.

Morphological analysis. Larval length was measured under a dissecting microscope and head capsule width (at the level of the eyes) and abdominal tubule lengths were measured using a microscope linked to an image-analysis system.

Chironomus were sorted according to larval type on the basis of the presence or absence, types and length of abdominal tubules using a dissecting microscope. Although there have been several attempts to classify *Chironomus* larvae into types based on the morphology of their tubules (Harnisch 1942; Andersen 1949; Lindeberg & Wiederholm 1979; Shobanov *et al.* 1996; Shobanov 2002), the definitions in these schemes have been inconsistent and in some cases contradictory. In general we have returned to the original scheme of Harnisch (1942), for which many of the types were well-illustrated by Andersen (1949). To this scheme we have introduced two additional types, bathophilus-type and melanotus-type, from the more recent scheme of Shobanov (2002). While the early schemes clearly recognised the coiled nature of the ventral tubules of *C. thummi* (now *C. riparius*) and *C. plumosus*, this aspect was lost in later classifications, such as those of Lindeberg and Wiederholm (1979) and Shobanov (2002), which were based only on the length of the tubules. We have found the distinction between coiled and relatively straight tubules to be consistent within species, and also a useful distinction among species. The bathophilus- and melanotus-types can fill this gap but only if the Shobanov (2002) definition is broadened to cover larvae with long ventral tubules, but without the typical coiling of those seen in thummi-type and plumosus-type. It should be noted that Shobanov (2002) introduced the melanotus-type to replace the anthracinus-type because *C. anthracinus* does not have an anthracinus-type larva (i.e. with lateral tubules), but is a typical thummi-type (i.e. without lateral tubules) (see larval description in the "Results and discussion" section). Further, the anthracinus-type, along with the semi-thummi- or semi-bathophilus-types, were intended to define in part larvae with very small lateral tubules, but we have found it difficult to draw a clear line between short and long lateral tubules and so have ignored this criterion. Our amended version of the larval classification is presented in Table 3.

TABLE 3. Classification of *Chironomus* larval types. See Fig. 1 for illustrations of ventral tubules.

Larval type	Pair of lateral tubules on 10th segment	Two pairs of ventral tubules on 11th segment[a]	
		Anterior pair	Posterior pair
salinarius	absent	absent	absent
halophilus	absent	absent or short	short
bathophilus	absent	straight; long	straight; long
fluviatilis[b]	absent	slightly curved, coming to a point at ends; long	slightly curved, coming to a point at ends; long
thummi	absent	with elbow; long	coiled; long
reductus	present	absent	absent
semireductus	present	straight; short	straight or may be slightly curved; short
melanotus	present	straight or slightly curved; long	straight or slightly curved; long
plumosus	present	with elbow; long	coiled; long

[a] long: ventral tubules $\geq$ the width of 11th segment

short: ventral tubules < the width of 11th segment

[b] Often hard to distinguish from bathophilus-type

FIGURE 1. Ventral tubules of the various larval types: salinarius and reductus (a), bathophilus and melanotus (b), fluviatilis (c), thummi and plumosus (d), as well as semireductus (e).

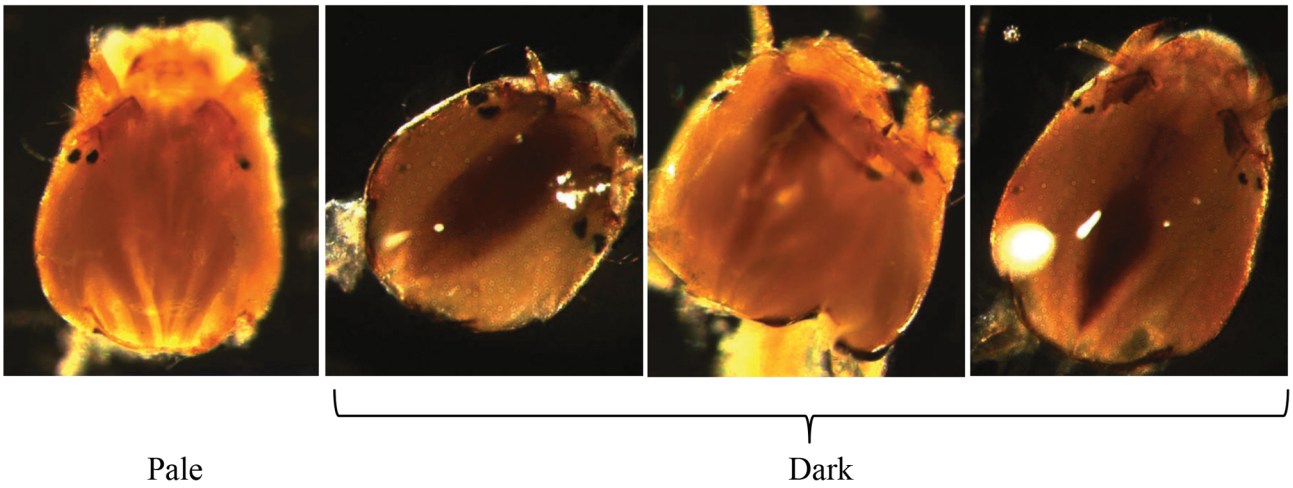


FIGURE 2. Dorsal view of larval head-capsules showing variation among species in the color of the frontoclypeus from pale (*C.* sp. NAIII) to dark (from left to right: *C. 'tigris'*, *C. cucini*, *C. dilutus*).

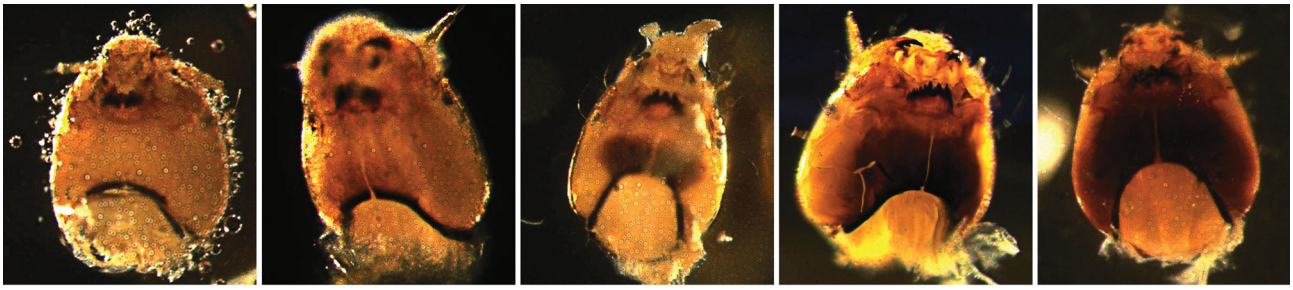


FIGURE 3. Ventral view of larval head-capsules showing variation among species in the color of the gula from pale (*C. maturus*) to slightly darkened (*C. harpi*), to posteriorly darkened (*C.* nr. *atroviridis* (sp. 2i)), to strongly darkened (*C. staegeri*), to completely darkened (*C. 'tigris'*).

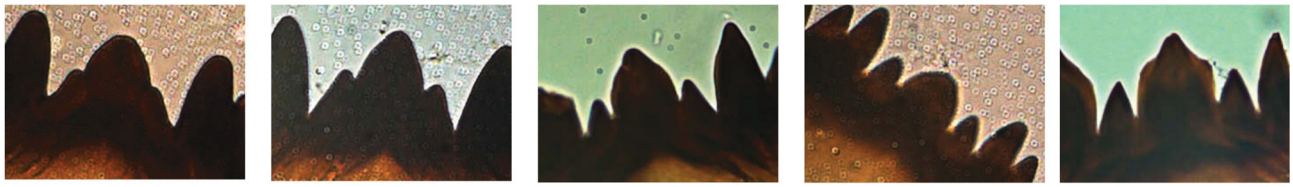


FIGURE 4. Types of mentum middle trifid tooth: (type A) *C.* nr. *atroviridis* (sp. 2i), (type B) *C. cucini*, (type C) *C. staegeri*, (type D) *C. maturus* (left) and *C. plumosus* (right).

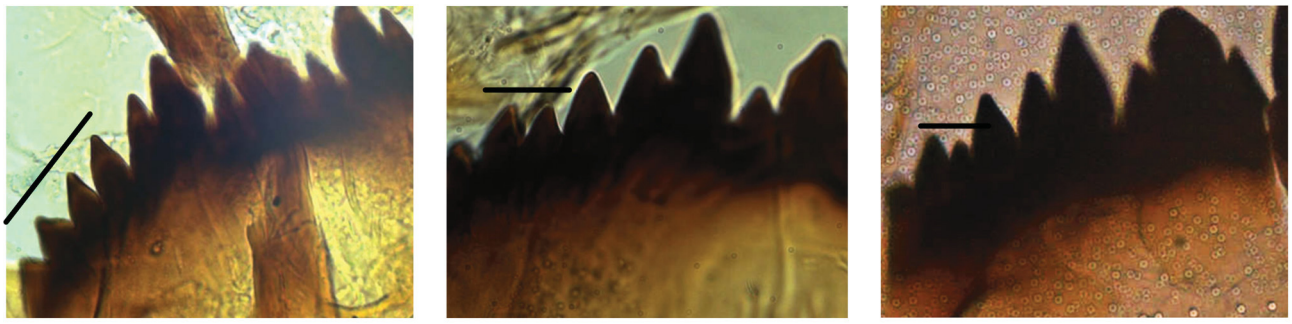


FIGURE 5. Types of mentum 4th lateral teeth (Webb & Scholl 1985; Vallenduuk & Moller Pillot 1997): (type I) *C. plumosus*, (type II) *C. staegeri*, (type III) *C. cucini.*

Head capsules were separated into parts and mounted in Canada Balsam so as to determine the coloration of the frontoclypeus (Fig. 2) and the gula (Fig. 3) as well as the structure of: the central trifid tooth (Fig. 4) and 4th lateral teeth of the mentum (Webb & Scholl 1985; Vallenduuk & Moller Pillot 1997) (Fig. 5), the mandibles (Fig. 6), the pecten epipharyngis (Fig. 7) and the ventromental plates (Webb *et al.* 1985). We developed a classification scheme based on differences in the central trifid tooth of the mentum (Fig. 4) and teeth of the mandibles (Fig. 6) that is based in part on the previous classifications of Webb and Scholl (1985) and Vallenduuk and Moller Pillot (1997), but that better encompasses the range in variation we observed in these structures. For example, Webb and Scholl (1985) classified the central trifid tooth of the larval mentum according to the degree of fusion of its three

component teeth, the width of the middle tooth, and the height of the outer teeth relative to the middle tooth. We found that the latter two criteria varied substantially within species and so considered only the first of these three criteria for that character. We note that although the degree of sharpness of the teeth of the mentum has been used for separating some *Chironomus* species (Martin 2013), this feature varied widely within the species under study and thus we did not use it for separating our study species. Lastly, we used the coloration of the 3rd inner mandibular tooth and its degree of fusion with the lower mandibular margin to classify larvae.

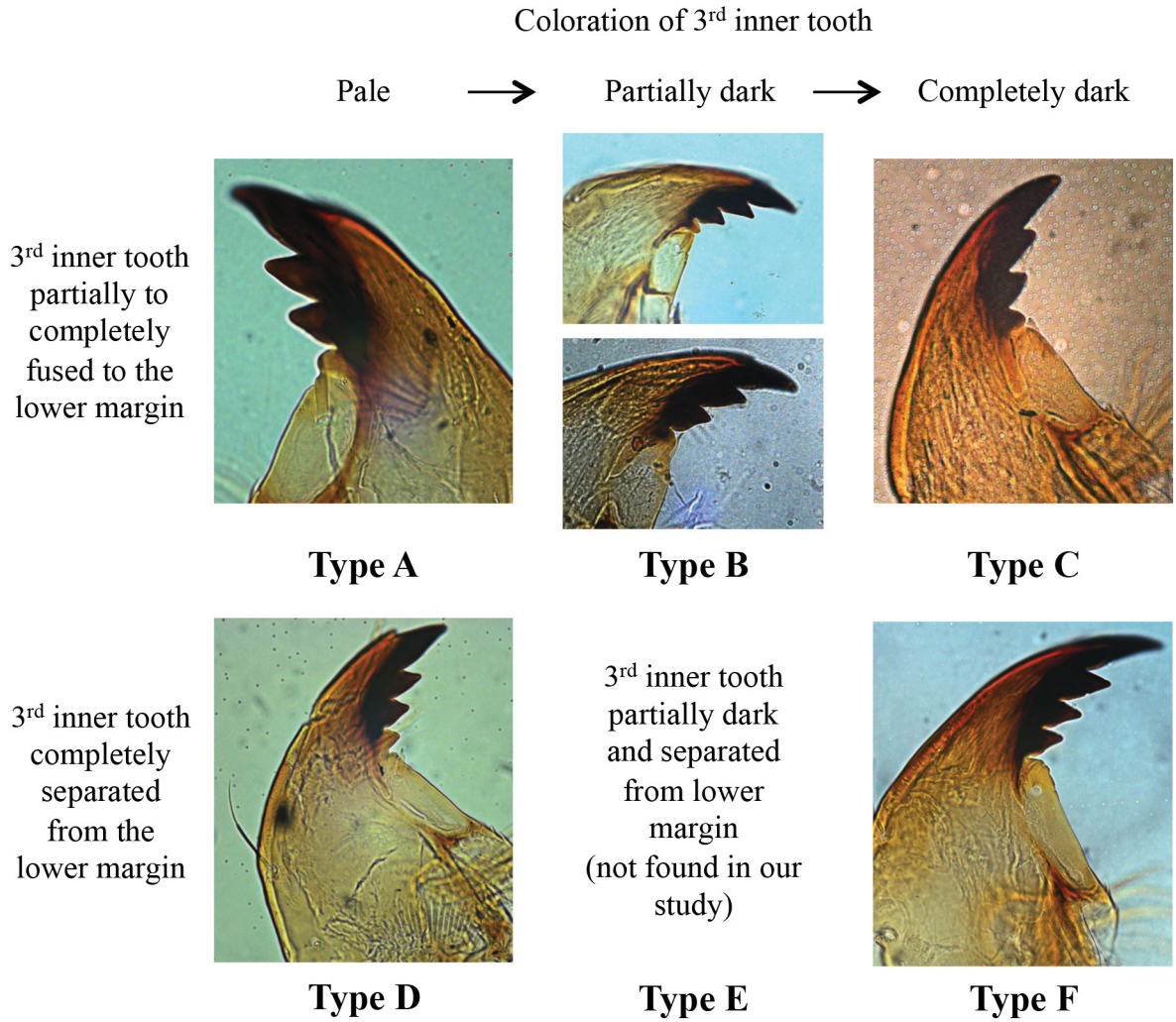
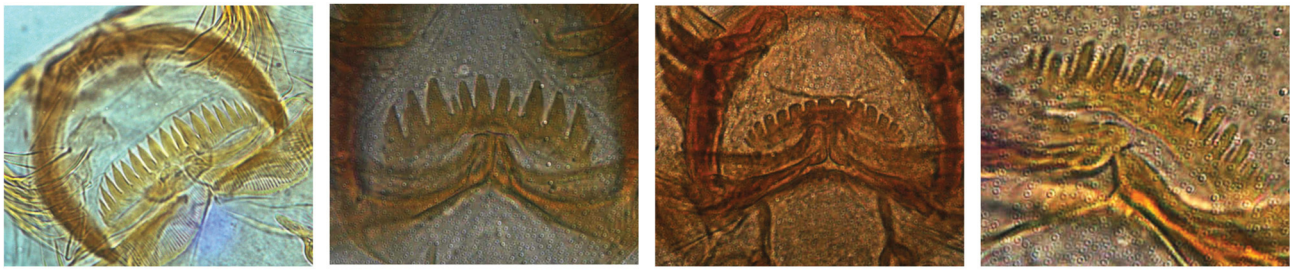


FIGURE 6. Mandible types as defined by the degree of darkening and separation of the 3rd inner tooth: (type A) *C. bifurcatus*, (type B) *C*. sp. NAI (above) and *C. ochreatus* (below), (type C) *C.* nr. *atroviridis* (sp. 2i), (type D) *C. staegeri*, (type F) *C. plumosus.*

Cytological analysis. Isolated salivary glands were prepared for polytene chromosome analysis using the aceto-orcein method (Martin *et al.* 2006). Veronika Golygina (Institute of Cytology and Genetics, Novosibirsk, Russia) assisted in distinguishing cytogenetically between *C. entis* and *C. plumosus*. Preparations of these two species have been deposited at her Institute. Polytene chromosome mounts of the remaining species have been deposited, together with their respective head capsule mounts, at the Canadian National Collection of Insects, Arachnids and Nematodes in Ottawa, Ontario, Canada.

Species delimitation and identification. Larvae were sorted according to their morphology, their *cox1* PCR-RFLP profiles (larvae collected in 2006 and 2007) and their *cox1* and *gb2β* gene partial nucleotide sequences and then linked, via DNA sequences of cytologically-known species (either already in GenBank, or from karyotyping larvae also sequenced in this study) to recognized species.



Type A
Pointed and rather uniform teeth

Type B
Pointed teeth with occasionally smaller teeth interspersed between larger teeth

Type C
Rounded and rather uniform teeth

Type D
Rounded teeth with smaller teeth interspersed between larger teeth

FIGURE 7. Types of teeth on the pecten epipharyngis: (type A) *C. staegeri*, (type B) *C. anthracinus*, (type C) *C. dilutus*, (type D) *C. ochreatus.*

Results and discussion

Species delimitation and identification

Species identifications are performed on 4th (final) instar larvae. We confirmed that *Chironomus* larvae were in the fourth instar by comparing the width of their head capsule (Table 6) to those of prepupal larvae and larval exuviae attached to pupae (data not shown). We did not measure the head capsule widths of *Chironomus* sp. NAII larvae, but these were undoubtedly 4th instars because we collected them just prior to adult emergence. Fourth instar larvae can also be recognized by the presence of developing imaginal discs in the thorax and/or posterior abdominal segments (Wülker & Götz 1968; Ineichen *et al.* 1983).

Analysis using PCR-RFLP of the *cox1* gene was performed on 296 larvae. The enzymes *Ssp* I, *Hinf* I, *Rsa* I and *Taq* I were used to cleave the partial *cox1* gene into different RFLP profiles (Table 4). These profiles were congruent with our groupings based on larval morphology (larval types and head-capsule features), with the exception of a single profile (*Ssp* I: 500,240; *Hinf* I: 710; *Rsa* I: 500,240; *Taq* I: 260,200,190) obtained for two larval types that differed in the coloration of their frontoclypeus. For these larvae, the partial *cox1* gene was cleaved with two additional restriction endonucleases, *Hha* I and *Alu* I, thereby creating three extra RFLP profiles. Results of these analyses are summarized in Table 4. To verify the accuracy of these results, larvae that included all of these RFLP profiles were sequenced.

We sequenced the partial *cox1* gene of 59 larvae that included all 15 RFLP profiles, as well as that of 79 other larvae (Fig. 8). We also amplified and sequenced the partial *gb2β*-gene of 83 larvae (Fig. 9). However, we were unsuccessful in obtaining the *gb2β* sequence for all *Chironomus* species (Table 4) despite modifying PCR conditions and testing several primer combinations (Table 2).

For visualisation purposes only, one representative of each unique sequence was used to illustrate the relationship between species in the *cox1* (Fig. 8) and *gb2β* ID-trees (Fig. 9). However, trees were also built using all sequences (including individuals that had identical gene sequences) which showed that using only unique sequences did not affect tree topology. Sequences were grouped into potential species according to molecular evidence (sequence clusters with bootstrap values >90% and sequence divergences of <4%) and larval morphology (see curly brackets in Figs. 8–9). Following this, species were identified through polytene chromosome analysis (71 larvae) and DNA barcoding. For cytological analyses, results of these identifications are given after the vertical line located to the right of the corresponding sequences in Figures 8 and 9. For DNA barcoding, Nearctic *Chironomus cox1*-sequences and *gb2β*-sequences from GenBank (http://www.ncbi.nlm.nih.gov) that grouped (bootstrap values >90% and sequence divergences <4%) with our sequences were added to our ID-trees. Moreover, we sequenced the *cox1* gene and/or the *gb2β* gene of voucher specimens (Table S2) and added these sequences to

TABLE 4. Summary of molecular results for the *cox1* and *gb2β* genes for each *Chironomus* species. *Cox1* gene: *cox1*-RFLP fragment sizes in base pairs (bp); 710-bp fragments are uncut; number of individuals analysed from real digests and number of individuals who subsequently had their *cox1* gene sequenced. *Gb2β* gene: primers used to amplify and sequence the *gb2β* gene; absence or presence of an intron (type I or type II) in the amplified *gb2β* sequence. ND; Not determined.

Species	*cox1* RFLP analysis: *cox1* RFLP fragment sizes in base pairs obtained from real digests (additional profiles obtained from simulation digests)						*cox1*		*gb2β*	
	Ssp I	*Hinf* I	*Rsa I*	*Taq* I	*Hha* I	*Alu* I	Number of individuals analysed from real digests	Number of individuals analysed through RFLP and subsequently sequenced	Primers used (refer to Table 2)	Intron: type or absence
C. anthracinus	710 (709)	710 (709)	710 (709)	380, 180, 100 (351, 174, 89, 72, 23)	ND	ND	16	3	wyk1b and wyk4	type II
C. bifurcatus (gr. 1)	710 (709)	510, 220 (489, 220)	610, 120 (596, 113)	550, 100 (525, 89, 72, 23)	(709)	(381, 214, 60, 33, 21)	12	4	wyk1b and wyk4	no intron
C. bifurcatus (gr. 2)	710 (709)	710 (709)	610, 120 (596, 113)	430, 100 or 550, 100 (426, 99, 89, 72, 23) or (525, 89, 72, 23)	(709)	(381, 150, 64, 60, 21, 18, 15 or 381, 214, 60, 21, 18, 15)	24	5	wyk1b and wyk4	no intron
C. cucini	710 (709)	710 (709)	460, 240 (443, 226, 40)	450, 100 (411, 99, 95, 89, 15)	ND	ND	26	5	wyk1b and wyk4	no intron
C. decorus-group sp. 2	710 (709)	710 (709)	500, 130 (483, 113, 113)	550, 100 (525, 95, 89)	ND	ND	10	3	no primer combination worked	ND
C. dilutus	(709)	(709)	(596, 87, 26) (683, 26)	(548, 89, 72) (533, 89, 72, 15)	(709)	(192, 150, 124, 93, 87, 63) (279, 150, 124, 93, 63)	0	0	wyk1b and wyk4	no intron
C. entis	710 (709)	450, 280 (439, 270)	500, 240 (483, 226)	250, 220, 100 (229, 208, 95, 89, 88)	ND	ND	1	0	wyk1b and wyk4	type II
C. frommeri	500, 240 (474, 235)	710 (709)	500, 240 (483, 226)	260, 200, 190 (252, 174, 99, 95, 89)	710 (709)	430, 220, 80 (414, 214, 81)	7	3	no primer combination worked	ND
C. harpi	(709)	(502, 207)	(330, 153, 113, 113)	(426, 194, 89)	ND	ND	0	0	wyk1b and wyk4	no intron

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TABLE 4. (Continued)

Species	*cox1* RFLP analysis								*gb2β*	
	cox1 RFLP fragment sizes in base pairs obtained from real digests (additional profiles obtained from simulation digests)						Number of individuals analysed from real digests	Number of individuals analysed through RFLP and subsequently sequenced	Primers used (refer to Table 3)	Intron: type or absence
	Ssp I	*Hinf I*	*Rsa I*	*Taq I*	*Hha I*	*Alu I*				
C. maturus	500, 240 (474, 235)	710 (709)	500, 240 (483, 226)	450, 100 (426, 99, 95, 89)	ND	ND	10	2	wyk1b and wyk4	no intron
C. nr. *atroviridis* (sp. 2i)	(709)	(709)	(683, 26)	(525, 95, 89)	(709)	(414, 177, 118)	0	0	primer combinations either failed or gave *gb7A*	ND
C. ochreatus	(709)	(439, 270)	(346, 337, 26)	(620, 89)	ND	ND	0	0	no primer combination worked	ND
C. plumosus	710 (709)	450, 280 (439, 270)	500, 240 (483, 226)	250, 220, 100 or 332, 220, 100 (229, 208, 95, 89, 88 / 317, 208, 95, 89 or 525, 95, 89)	ND	ND	8	2	wyk1b and wyk4	type II
C. sp. NAI	710 (709)	710 (709)	710 (709)	260, 180, 100 (252, 174, 99, 89, 72, 23)	ND	ND	9	2	wyk1b and wyk4	type II
C. sp. NAII	710 (709)	510, 220 (502, 207)	500, 240 (483, 226)	470, 180 (446, 174, 89)	ND	ND	4	2	no primer combination worked	ND
C. sp. NAIII	500, 240 (474, 235)	710 (709)	710 (709)	450, 210, 100 (426, 99, 95, 89)	ND	ND	45	6	wyk1b and *2β*-A	type II
C. staegeri	500, 240 (474, 235)	710 (709)	500, 240 (483, 226)	260, 200, 190 (252, 194, 174, 89)	330, 440 (389, 320)	400, 130, 80 (378, 124, 90, 81, 36)	37	8	no primer combination worked	ND
C. 'tigris'	500, 240 (474, 235)	710 (709)	500, 240 (483, 226)	260, 200, 190 (252, 194, 174, 89)	710 (709)	430, 170, 120 (414, 171, 124 / 381, 171, 124, 33 or 255, 171, 159, 124)	61	12	wyk1b and wyk4	type II

TABLE 5. Average (%) pairwise sequence divergence between species and within species (given diagonally in bold font) for the *cox1* and *gb2β* genes. For each species and each gene, the number of sequences analysed is given in parentheses. Interspecific sequence divergences within the intraspecific range of *Chironomus* species (*cox1*: 0-3% and *gb2β*: 0-2%; see Table S3) are highlighted in grey.

	C. anthracinus		*C. bifurcatus*		*C. cucini*		*C. decorus*-group sp. 2		*C. dilutus*		*C. entis*	
	cox1 (16)	*gb2β* (6)	*cox1* (25)	*gb2β* (10)	*cox1* (7)	*gb2β* (4)	*cox1* (10)	*gb2β* (0)	*cox1* (11)	*gb2β* (11)	*cox1* (11)	*gb2β* (11)
C. anthracinus	**0.06**	**0.34**										
C. bifurcatus	15.21	30.34	**1.24**	**0.29**								
C. cucini	14.07	22.69	13.10	28.40	**0.21**	**0.21**						
C. decorus-group sp. 2	15.19		9.88		14.35		**0.26**					
C. dilutus	16.21	24.74	15.57	29.16	15.00	24.73	14.38		**1.34**	**0.00**		
C. entis	13.48	10.02	16.60	32.66	15.21	22.30	15.44		15.90	26.45	**1.30**	**0.25**
C. frommeri	13.26		13.20		11.89		10.90		15.80		12.91	
C. harpi	15.98	32.60	11.99	17.50	16.35	25.41	11.37		16.12	33.73	18.67	32.17
C. maturus	15.72	17.44	12.94	27.00	14.45	19.60	11.15		13.86	16.08	15.03	19.30
C. nr. *atroviridis* (sp. 2i)	19.04		15.93		16.17		14.85		14.44		17.70	
C. ochreatus	18.86		17.47		16.17		15.20		15.41		17.70	
C. plumosus	13.38	13.35	16.33	33.27	15.01	24.93	15.36		15.74	37.07	1.09	14.97
C. sp. NAI	4.48*	2.88*	15.47	32.39	14.73	26.05	15.47		17.79	28.72	14.01	13.09
C. sp. NAII	14.37		16.85		14.65		14.34		16.60		14.50	
C. sp. NAIII	16.43	35.27	18.03	31.82	15.54	18.00	15.55		18.87	28.64	19.45	32.61
C. staegeri	14.73		14.53		13.14		12.05		15.97		13.30	
C. 'tigris'	14.20	28.50	14.23	34.71	12.06	23.11	11.69		15.90	27.36	12.58	27.96

*Sequence interspecific divergences range from 1 to 5%.

TABLE 5. (Continued)

	C. frommeri		*C. harpi*		*C. maturus*		*C.* nr. *atroviridis* (sp. 2i)		*C. ochreatus*		*C. plumosus*	
	cox1 (3)	*gb2β* (0)	*cox1* (6)	*gb2β* (6)	*cox1* (3)	*gb2β* (1)	*cox1* (6)	*gb2β* (0)	*cox1* (3)	*gb2β* (0)	*cox1* (9)	*gb2β* (29)
C. frommeri	**0.41**											
C. harpi	14.83		**0.22**	**0.00**								
C. maturus	11.80		13.40	31.98	**0.21**							
C. nr. *atroviridis* (sp. 2i)	15.43		18.01		13.97		**1.33**					
C. ochreatus	15.12		19.55		16.04		12.14		**0.10**			
C. plumosus	12.70		18.48	28.69	14.73	27.49	17.54		17.59		**0.93**	**0.64**
C. sp. NAI	13.31		16.32	34.20	16.03	22.17	18.37		19.31		13.90	14.69
C. sp. NAII	12.34		14.89		13.35		17.88		17.26		14.58	
C. sp. NAIII	13.57		16.55	30.05	13.96	22.89	18.10		17.58		19.27	34.09
C. staegeri	3.40		14.36		12.55		16.68		15.85		13.19	
C. 'tigris'	2.68		14.75	35.83	12.38	18.48	16.47		15.66		12.44	33.11

TABLE 5. (Continued)

	C. sp. NAI		*C.* sp. NAII		*C.* sp. NAIII		*C. staegeri*		*C. 'tigris'*	
	cox1 (3)	*gb2β* (3)	*cox1* (2)	*gb2β* (0)	*cox1* (13)	*gb2β* (4)	*cox1* (16)	*gb2β* (0)	*cox1* (17)	*gb2β* (4)
C. sp. NAI	**0.82**	**0.00**								
C. sp. NAII	15.75		**0.77**							
C. sp. NAIII	16.54	37.28	14.40		**0.35**	**0.00**				
C. staegeri	14.79		13.49		13.94		**0.04**			
C. 'tigris'	14.24	30.69	12.76		13.33	15.01	1.49		**0.15**	**0.00**

TABLE 6. Larval morphology of fourth-instar larvae of the various *Chironomus* species collected in this study.

Species	n	Larval type (Table 3 and Fig. 1)	Mean tubule length in mm (range)			Mean larval length in mm (range)	Head width in mm (range)	Frontoclypeus color (Fig. 2)	Gula color (Fig. 3)	Mentum type		Mandible type	Pecten epipharyngis		Anterior margin of ventromental plates
			Lateral tubules	Anterior ventral tubules	Posterior ventral tubules					Central trifid tooth (Fig. 4)	4th lateral teeth (Fig. 5)	3rd inner tooth (Fig. 6)	Mean no. of teeth (range)	Type (Fig. 7)	
C. cucini	26–31	salinarius	absent	absent	absent	18 (15–21)	0.54 (0.49–0.62)	pale or slightly darkened with lobed dark spot anteriorly	posteriorly darkened	B	III	A	15 (12–19)	B	smooth
C. sp. NAII	4	salinarius	absent	absent	absent	12 (11–13)	not measured	dark longitudinal stripe with lobed dark spot medially	posteriorly darkened	C	II	B	13 (12–14)	A	smooth
C. sp. NAIII	47–52	salinarius	absent	absent	absent	14 (10–18)	0.48 (0.43–0.54)	pale	posteriorly to strongly darkened	B	II or III	A	12 (9–15)	A	smooth
C. bifurcatus	14–50	bathophilus	absent	1.5 (0.9–1.9)	1.2 (1.0–1.7)	13 (11–16)	0.49 (0.42–0.59)	pale	posteriorly to strongly darkened	B	II	A	13 (10–16)	A	smooth
C. decorus-group sp.2	5–17	bathophilus, fluviatilis	absent	0.9 (0.7–1.5)	0.9 (0.5–1.4)	14 (11–16)	0.56 (0.51–0.60)	pale	strongly to completely darkened	B or C	II	A	15 (13–20)	A	smooth
		melanotus	0.18 (0.16–0.21)												
C. anthracinus	22–27	thummi	absent	1.3 (1.0–1.6)	1.0 (0.6–1.5)	18 (13–21)	0.64 (0.60–0.67)	pale	strongly to completely darkened	B or C	II	A or B	16 (13–18)	B	relatively smooth
C. nr. *atroviridis* (sp.2i)	4	thummi	absent	1.0 (1.0–1.0)	0.9 (0.9–1.0)	19 (17–20)	0.57 (0.56–0.57)	pale	darkened posteriorly	A	I	C	16 (14–16)	D	smooth

……continued on the next page

TABLE 6. (Continued)

Species	n	Larval type (Table 3 and Fig. 1)	Mean tubule length in mm (range)			Mean larval length in mm (range)	Head width in mm (range)	Frontoclypeus color (Fig. 2)	Gula color (Fig. 3)	Mentum type		Mandible type	Pecten epipharyngis		Anterior margin of ventromental plates
			Lateral tubules	Anterior ventral tubules	Posterior ventral tubules					Central trifid tooth (Fig. 4)	4th lateral teeth (Fig. 5)	3rd inner tooth (Fig. 6)	Mean no. of teeth (range)	Type (Fig. 7)	
C. ochreatus	2	thummi	absent	1.0 (1.0–1.1)	0.9 (0.7–1.2)	not measured	0.52 (0.51–0.52)	pale	slightly darkened	B	I	B	23 (22–24)	D	smooth
C. sp. NAI	9	thummi	absent	1.4 (1.2–1.8)	1.1 (0.9–1.7)	18 (16–21)	0.64 (0.64)	pale	strongly to completely darkened	B or C	II	A or B	15 (10–18)	B	relatively smooth
C. entis	5–8	semireductus	0.26 (0.21–0.32)	0.5 (0.4–0.8)	0.6 (0.3–0.9)	25 (22–28)	0.83 (0.78–0.85)	pale	strongly to completely darkened	C or D	I	F	14 (13–17)	B	smooth to slightly crenulated
C. plumosus	14–33	semireductus or plumosus	0.35 (0.18–0.49)	1.0 (0.5–1.7)	0.9 (0.4–1.8)	22 (13–26)	0.75 (0.65–0.89)	pale	strongly to completely darkened	C or D	I	F	16 (12–21)	B	smooth to slightly crenulated
C. dilutus	9	plumosus	0.47 (0.32–0.72)	1.8 (1.4–2.7)	1.8 (1.3–2.6)	24 (17–28)	0.67 (0.61–0.73)	dark, particularly in the center posteriorly	slightly to posteriorly darkened	C	I	B	13 (10–15)	A or C	smooth
C. frommeri	7	plumosus	0.52 (0.42–0.63)	3.4 (2.7–4.1)	3.7 (3.1–4.6)	22 (19–24)	0.64 (0.59–0.64)	pale	strongly to completely darkened	C	II	A or D	15 (13–18)	A	crenulated
C. harpi	2–6	plumosus	0.17 (0.15–0.22)	1.1 (0.9–1.3)	1.2 (0.9–1.6)	12 (10–14)	0.40 (0.38–0.42)	pale	slightly darkened	B	II	A	15 (14–15)	B	smooth
C. maturus	10	plumosus	0.40 (0.32–0.44)	2.9 (2.3–3.3)	2.8 (2.2–3.5)	18 (14–21)	0.52 (0.49–0.56)	pale	pale to slightly darkened	D	I	D	20 (18–20)	B	smooth
C. staegeri	30–44	plumosus	0.34 (0.23–0.55)	1.6 (0.9–3.7)	1.7 (1.0–3.8)	20 (14–28)	0.70 (0.62–0.76)	pale	strongly to completely darkened	C	II	A or D	17 (12–20)	A	crenulated
C. 'tigris'	42–66	plumosus	0.18 (0.11–0.25)	1.7 (0.9–2.5)	1.4 (0.8–2.1)	16 (9–22)	0.61 (0.54–0.68)	dark	strongly to completely darkened	C or D	II	A or D	15 (10–20)	B	relatively smooth

our ID-trees (Figs. 8–9). *C. acidophilus* (Keyl 1960), *C. calligraphus* (Goeldi 1905), *C. quinnitukqut* (Martin *et al.* 2010), *C*. sp. g (Martin 2013), *C*. sp. h (Martin 2013) and *C*. sp. u (Martin 2013) did not group with any of our collected species, so we could rule out these species as being any of our unrecognized species. Analysis of all the sequences together with simulation digests allowed us to identify the *Chironomus* species that had been previously separated using RFLP analysis. Simulation digests were performed on all of the *cox1* sequences obtained (see Table 4). Extra RFLP profiles were obtained from these simulation digests.

On the basis of the genetic, morphological and cytological information that we obtained, we conclude that the 404 *Chironomus* larvae that we collected represent 17 species, 14 of which were known while the status of three others remains uncertain. A detailed list of all the larvae analysed is presented in Table S1. In the following section, pertinent genetic, morphological and cytological information is presented for each of these species in the order that they are presented in Figure 8 (from top to bottom). Detailed genetic information, including *cox1*-RFLP sizes, the primers used to amplify the *gb2β* gene and whether or not the *gb2β* gene was amplified is given in Table 4. The *gb2β* gene of some *Chironomus* species includes an intron (type I or type II), whereas in others it is absent (Hankeln *et al.* 1997; Makarevich *et al.* 2000). This information is also given in Table 4. The intraspecific and interspecific divergences in *cox1* and *gb2β* sequences are summarized in Table 5, with more detail presented in Table S3. Overall, intraspecific divergences for *Chironomus* species characterized by *cox1* range between 0 and 3%, whereas intraspecific sequence divergences based on *gb2β* ranged between 0 and 2%. A detailed morphological description for each species is found in Table 6. Where pertinent, we also discuss the status of each species collected and its relationship to other closely-related species that we did not collect in our study. Following our species descriptions, we present a morphological key to discriminate among larvae of the 17 *Chironomus* species that we collected. Species from our study area (Ontario and Quebec) that are not included in the key are given in Martin (2013). Lastly, the distribution and ecology of each of the *Chironomus* species that we collected are summarized in Table 7.

TABLE 7. Lakes in which the various *Chironomus* species were collected as well as lake characteristics including sampling depth, pH and trophic status. Lake codes are given in Table 1. ND; not determined.

Species	Depth (m)	pH	Trophic status	Water body
C. anthracinus	2–12	4.5–8.5	oligo to mesotrophic	AR, HA, OS, PI, RA, RM, SI (2011)
C. bifurcatus	1.5–24	2.7–7.8	oligo to eutrophic	AD, AR, DA, DP, KI, MC, OP, SJ, TI (2011)
C. cucini	9–35	6.2–7.5	oligo to mesotrophic	BO, CL, OP, SJ, VA
C. decorus-group sp.2	1–5	7.2–7.6	oligo to mesotrophic	AD, DF, DP, FO, OP, SC, SI (2011)
C. dilutus	1.5–5	7.5–8.4	eutrophic	KE
C. entis	1–9	7.1–8.3	meso to eutrophic	DA, DS, MN, OP, PE,
C. frommeri	1	ND	ND	PO
C. harpi	1–4	2.7–3.8	oligotrophic	AR
C. maturus	4–6	ND	ND	BE
C. nr. a*troviridis* (sp. 2i)	1	7.4	meso to eutrophic	MN
C. *ochreatus*	3	7.7	mesotrophic	OP
C. plumosus	1–8	6.8–8.5	oligo to eutrophic	AU, DA, DP, FO, KE, KI, MN, OS, PE, RO
C. sp. NAI	7.5	6.8	oligotrophic	KA
C. sp. NAII	4	5.9	oligotrophic	SI (2007)
C. sp. NAIII	5–12	7.1–7.9	oligo to mesotrophic	DA, HA, MC, RA, RM
C. *staegeri*	1–10	5.9–8.0	oligo to eutrophic	CR, DA, DP, KA, KI, MC, OP, PO, SI (2007), SJ, TI (2007 and 2011)
C. '*tigris*'	2–10	5.9–8.0	oligo to mesotrophic	KA, MC, OP, SI (2007), SJ, TI (2007 and 2011)

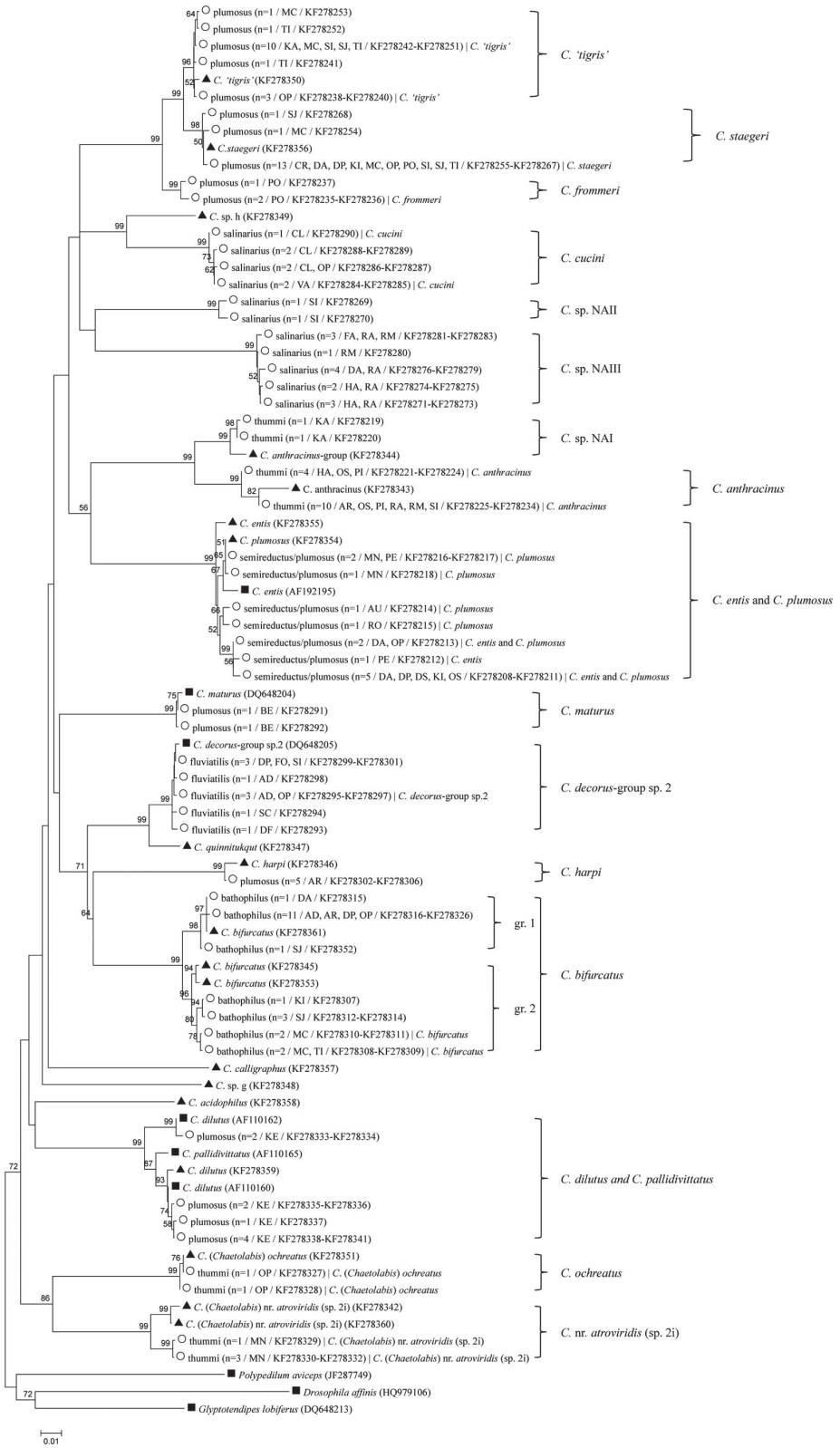

FIGURE 8. Neighbor-joining identification tree (NJ ID-tree) based on partial *cox1* sequences and the K2P substitution model. Numbers on branches are bootstrap values >50%. ○ Sequences of *Chironomus* species collected from lakes in our study. Larval morpho-types are specified followed in parenthesis by: sample size (n = the number of individuals sequenced for each consensus sequence), lake abbreviations, and GenBank accession numbers. Some larvae were identified by examining their polytene chromosomes, and these results are indicated alongside the corresponding sequence next to the vertical line. ■ Sequences obtained from GenBank (species name and GenBank accession number in parenthesis). ▲ Sequences obtained from cytologically identified reference *Chironomus* specimens (species name and GenBank accession number in parenthesis).

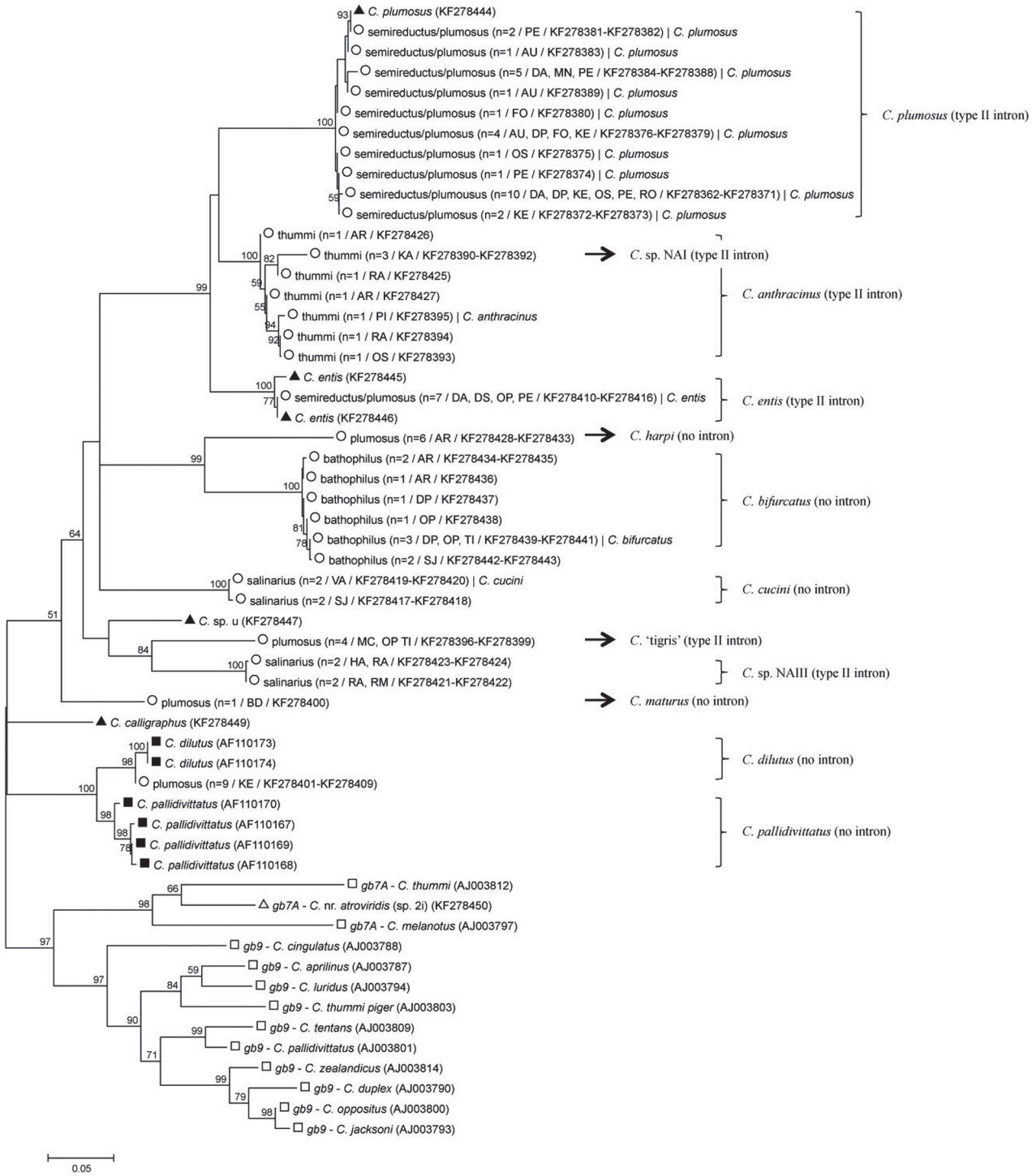

FIGURE 9. Neighbor-joining identification tree (NJ ID-tree) based on partial *gb2β* sequences and the K2P substitution model. ○ Sequences of *Chironomus* species collected from lakes in our study. Larval morpho-types are specified followed in parentheses by: sample size (n = the number of individuals sequenced for each consensus sequence), lake abbreviations and GenBank accession numbers. Some larvae were identified by examining their polytene chromosomes, and these results are indicated alongside the corresponding sequence next to the vertical line. ■ Sequences obtained from GenBank (species name and GenBank accession number in parenthesis). ▲ Sequences obtained from cytologically identified reference *Chironomus* specimens (species name and GenBank accession number in parenthesis). □ *Chironomus gb7A* and *gb9* sequences obtained from GenBank were also added as outgroups (globin name, species name and GenBank accession number in parenthesis). △*Chironomus gb7A* sequence obtained from cytologically identified reference specimens (globin name, species name and GenBank accession number in parenthesis).

Species descriptions and taxonomic status

Chironomus* (*Chironomus*) *'tigris'
(nomen nudum in Martin *et al.* (2008), for species *C*. sp. Am1 of Kiknadze *et al.* (1993)).

Material examined (Table S1): 66 larvae from Kasten Lake, McFarlane Lake, Silver Lake and Tilton Lake in Ontario as well as from Lake Opasatica and Lake St. Joseph in Quebec.

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both the *cox1* and *gb2β* genes. Compared to the other *Chironomus* species (except *C*. sp. NAIII), the *gb2β* gene of *C. tigris* is 3 codons short immediately after the end of the 2nd intron. *C. 'tigris'* sequences form distinct clades in both the *cox1* and *gb2b* ID-trees. Consequently either gene can be used to accurately separate and identify *C. 'tigris'*. However, the range of interspecific divergence between the *cox1*-sequences of *C. 'tigris"* and *C. staegeri* (1–2%), as well as between *C. 'tigris'* and *C. frommeri* (2–3%), are within the intraspecific sequence divergence range of collected and reference *Chironomus* species (0–3%). Therefore, *cox1* sequence divergence values alone cannot be used to reliably separate *C. 'tigris'* from *C. staegeri* or *C. frommeri*. For the *gb2b* gene, we could not assess the interspecific sequence divergence between *C. 'tigris'*, *C. staegeri* and *C. frommeri* because we were unsuccessful in amplifying the *gb2β* gene for *C. staegeri* and *C. frommeri*. In the *cox1* ID-tree, sequences of collected larvae cluster with the reference sequence of *C. 'tigris'*, thus confirming the identification of this species. Chosen restriction enzymes for the *cox1* PCR-RFLP analysis correctly separated *C. 'tigris'* larvae from the other *Chironomus* species.

Morphology (Table 6). Our specimens are large sized plumosus-type larvae having their anterior ventral tubules longer than the posterior ventral tubules. The frontoclypeus of *C. 'tigris'* is dark-colored, which distinguishes it from larvae of the otherwise morphologically-similar *C. staegeri* and *C. frommeri*, which both have a pale frontoclypeus. Exceptionally, in larvae from some other regions, the frontoclypeus of *C. staegeri* is reported to be slightly darkened (Martin 2013). However, this criterion could prove to be less clear cut and other morphological features such as the length of the lateral tubules and the anterior margin of ventromental plates could be used to separate these species.

Cytology. The cytology of the two larvae analyzed clearly indicates that this species is *C. 'tigris'* since it is one of only two *Chironomus* species known to possess two polytene chromosomes. In this respect, it is clearly distinct from *C. staegeri* and *C. frommeri* which possess three and four chromosomes, respectively. The arm combination of *C. 'tigris'* chromosomes is GAB, FEDC and its chromosomes are described in Martin *et al.* (1974), Butler *et al.* (1995, *C*. sp. r), Kiknadze *et al.* (1993, *C*. sp. Am1) and Martin (2013).

Distribution and ecology (Table 7). This species has been previously reported from lakes in Minnesota, Ontario, Quebec and Wisconsin (Butler *et al.* 1995; Martin *et al.* 2008; Martin 2013). We found *C. 'tigris'* in oligotrophic to mesotrophic lakes of pH 5.9–8.0. At all sites where *C. 'tigris'* was collected, *C. staegeri* was also present. However, the reverse was not necessarily the case. Lakes in which *C. staegeri* was present and *C. 'tigris'* was absent tended to be eutrophic (with the exception of Crooked Lake), which suggests that *C. 'tigris'* larvae prefer less productive systems. Their northerly distribution in North America may reflect this fact. We collected *C. 'tigris'* at water depths varying from 2–10 m, although it can live at greater depths (20 m; Butler *et al.* 1995).

***Chironomus* (*Chironomus*) *staegeri* Lundbeck (1898)**

Material examined (Table S1): 44 larvae from Lake D'Alembert, Lake Duprat, Lake Kinojévis, Lake Opasatica, Lake St. Joseph and an unnamed pond in Quebec as well as from Crooked Lake, McFarlane Lake, Kasten Lake, Silver Lake, and Tilton Lake in Ontario.

DNA (Figs. 8–9 and Tables 4–5, S3). In the *cox1* ID-tree, *C. staegeri* sequences form a distinct clade. Sequences of collected larvae cluster with the reference sequence of *C. staegeri*, thereby confirming the identification of this species. We were not successful in amplifying the *gb2β* gene for this species. C*ox1* interspecific sequence divergences between *C. staegeri* and *C. 'tigris'* (1–2%) as well as *C. staegeri* and *C. frommeri* (3–4%) are within the intraspecific divergence range of *Chironomus* species assessed in this study (0–3%). Therefore, *cox1* sequence divergence values cannot be used to separate *C. staegeri* from *C. 'tigris'* or from *C. frommeri*. Restriction enzymes for the *cox1* PCR-RFLP analysis correctly separated *C. staegeri* from the other *Chironomus* species.

Morphology (Table 6). Our specimens of *C. staegeri* are large sized plumosus-type larvae with a pale frontoclypeus. This latter feature clearly distinguishes them from *C. 'tigris'* larvae that have a dark frontoclypeus. Outside of our study area, some *C. staegeri* larvae are reported to have a slightly darkened frontoclypeus (Martin 2013). Thus other features such as the length of the lateral tubules and the outline of the anterior margin of ventromental plates could be examined to separate these two species. In our study area, *C. staegeri* could be distinguished from *C. frommeri* by the fact that the ventral and lateral tubules of the former were about half the length of those of the latter (Table 6). However, tubule length is not likely a reliable character to separate these species because in the study by Sublette and Sublette (1971) tubule lengths overlapped between these species. Sublette and Sublette (1971) suggested that *C. frommeri* and *C. staegeri* larvae could be separated by the structure of the anterior margin and apex of their paralabial plates as well as the shape of the teeth of the pecten epipharyngis. However, these features did not reliably separate these species in our study area.

Cytology. The cytology of the single larva that we examined indicates that this species is *C. staegeri* since it has three chromosomes with a modified thummi arm combination of AB, CD, GEF (Wülker & Martin 1971; Kiknadze *et al.* 2004; Kiknadze *et al.* 2010). Thus it is distinct from *C. 'tigris'*, which possesses 2 chromosomes, and from *C. frommeri*, which has 4 chromosomes.

Distribution and ecology (Table 7). *C. staegeri* has been found in a variety of lentic habitats from deep lakes to shallow pools (Wülker *et al.* 1971) throughout Canada (British Columbia, Manitoba, Newfoundland, Northwest Territories, Ontario and Saskatchewan) and the United States (Alabama, California, Idaho, Illinois, Iowa, Kansas, Louisiana, Massachusetts, Michigan, Minnesota, Missouri, New Hampshire, New Jersey, New Mexico, New York, North Carolina, North Dakota, Pennsylvania, South Carolina, South Dakota, Tennessee, Washington and Wisconsin) (Sublette & Sublette 1971; Oliver *et al.* 1990; Martin *et al.* 2008; Martin 2013). In our study, *C. staegeri* was found in oligotrophic to eutrophic lakes and in a pond at depths ranging from 1–10 m, and at pH values ranging from 5.9–8.0.

Taxonomic comment. Given the polymorphism of chromosomal inversions in populations of *C. staegeri* in Canada and the Unites States, Martin and Wülker (1971) speculated that *C. staegeri* might be in the process of splitting into three species based in part on their restriction to waters of different depths. Our DNA data do not support this idea since there is little variation in the *cox1* nucleotide sequences between *C. staegeri* that we collected from a pond, and over a range of depths in several lakes. In fact, the mean *cox1* intraspecific divergence among *C. staegeri* sequences is very low (0.04%). The different distributions of chromosomal inversions might therefore be due to populations with different inversion sequences adapting to different ecological niches, as has been suggested for species such as *C. plumosus* (Butler *et al*. 1999).

Chironomus (*Chironomus*) *frommeri* Sublette and Sublette (1971)

Material examined (Table S1): 7 larvae collected from an unnamed pond on a military base near Trois-Rivières, Quebec.

DNA (Figs. 8–9 and Tables 4–5, S3). In the *cox1* ID-tree, *C. frommeri* nucleotide sequences form a distinct clade. However, as mentioned above, *cox1* sequence divergences between *C. frommeri* and *C. 'tigris'* (2–3%) as well as *C. frommeri* and *C. staegeri* (3–4%) are within the intraspecific sequence divergence range of *Chironomus* species assessed in this study (0–3%). Therefore, *cox1* sequence divergence values cannot be used to separate *C. frommeri* from *C. 'tigris'* or *C. staegeri*. We were not successful in amplifying the *gb2β* gene for this species. Chosen restriction enzymes for the *cox1* PCR-RFLP analysis correctly separated *C. frommeri* from the other *Chironomus* species.

Morphology (Table 6). Our specimens are large sized plumosus-type larvae with posterior ventral tubules longer than the anterior ones. Morphologically, larvae of *C. frommeri* from our study area can be distinguished from those of *C. 'tigris'* and *C. staegeri* (however, see comments above on the morphology of these species).

Cytology. The cytology of the larva analyzed permitted us to identify this species as *C. frommeri*. *C. frommeri* has four polytene chromosomes with the thummi arm combination of AB, CD, EF, G (Wülker & Martin 1971) as opposed to *C. 'tigris'* and *C. staegeri* that possess only 2 and 3 chromosomes, respectively. Arm G homologs of *C. frommeri* are closely paired, with a virtually terminal nucleolus, similar to the fused arm G of *C. crassicaudatus* (not collected in our study) (Wülker & Martin 1971). There is also another nucleolus proximal in arm B.

Distribution and ecology (Table 7). We were surprised to collect *C. frommeri* in eastern Canada because all previous collections of this species are from the western United States (California, Oregon, Utah and New Mexico) (Wülker *et al.* 1971; Oliver *et al.* 1990). A possible explanation for this apparent anomaly is that this species was transported from the west to the east via military equipment since it was collected in a pond located in a military base. This species is known to occur in lakes, oxbows and permanent ponds (Martin 2013).

Chironomus (*Chironomus*) *cucini* Webb (1969)

Material examined (Table S1): 31 larvae collected in lakes in Quebec (Lake Bousquet, Lake Opasatica, Lake St. Joseph and Lake Vaudray) and in Ontario (Clearwater Lake).

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both the *cox1* and *gb2β* genes. In both of these ID-trees, *C. cucini* forms a distinct cluster. Consequently both genes can be used to accurately separate and identify *C. cucini*. The *gb2β* gene sequence of *C. cucini* has no intron, but contains three extra base pairs at the 3' end of the sequence. Chosen restriction enzymes for the *cox1* PCR-RFLP analysis correctly separated *C. cucini* from the other *Chironomus* species.

Morphology (Table 6). Our specimens are large sized salinarius-type larvae lacking lateral and ventral tubules. In other study areas, larvae of *C. cucini* are reported to occasionally have small posterior ventral tubules (Wülker & Butler 1983). Morphologically, larvae of *C. cucini* are similar to those of *C*. sp. NAIII with two minor differences. First, the structure of the pecten epipharyngis differs slightly between the species (Table 6). Second, the mean (± 95% CI) ratio of the lengths of antennal segments 1 to 2–5 (AR) of *C*. sp. NAIII larvae (1.77 ± 0.08) was significantly lower than that of *C. cucini* (2.04 ± 0.08). Note however that there was overlap in the ranges of the ARs between the two species.

Cytology. The cytology of the 5 larvae analyzed permitted us to identify this species as *C. cucini*. *C. cucini* has four polytene chromosomes attached together by a chromocenter and with the thummi arm combination of AB, CD, EF, G (Martin 1979; Wülker & Butler 1983). In most populations, a single nucleolus is located in arm G, although in some California populations there is a second nucleolus in arm B.

Distribution and ecology (Table 7). *C. cucini* has been reported from across the Nearctic region (British Columbia, California, Indiana, Minnesota, New York and Ontario) (Oliver *et al.* 1990; Martin 2013). In our study area, *C. cucini* was found in the profundal zone of circum-neutral (pH 6.2–7.5), oligotrophic to mesotrophic, lakes.

Chironomus sp. NAII

Material examined (Table S1): 4 larvae from Silver Lake (Ontario).

DNA (Figs. 8–9 and Tables 4–5, S3). In the *cox1* ID-tree, *C.* sp. NAII nucleotide sequences clearly form a distinct clade. We were not successful in amplifying the *gb2β* gene for this species. Available DNA barcodes for *Chironomus* species did not cluster with those of *C.* sp. NAII. *Cox1* PCR-RFLP analysis correctly separated *C.* sp. NAII from the other *Chironomus* species.

Morphology (Table 6). Our specimens are medium sized salinarius-type larvae. Morphologically, larvae of *C*. sp. NAII closely resemble those of *C. cucini* and *C*. sp. NAIII, but differ by the lobed dark spot in the middle of its frontoclypeus, by the partial coloration of its 3rd mandibular tooth and by the type of mentum central trifid tooth.

Cytology. This species has four polytene chromosomes with two nucleoli, one of which is in arm G. The cytology does not correspond to any known salinarius-type specimens from North America or Europe. The main difference between *C*. sp. NAII and the other known salinarius-type species is its lack of heterochromatic centromeres and its banding sequence in arm G. The cytological preparations were generally too poor to determine further details. This may be the larva of a previously described northern *Chironomus* species, for which the larva is currently unknown, or it may be a completely new species.

Distribution and ecology (Table 7). This species was found in oligotrophic Silver Lake in 2007 at a depth of 4 m. In 2010 and 2011, we sampled the lake again in an effort to collect additional *C.* sp. NAII larvae. However, they were no longer present, perhaps because the pH of this lake had increased from 5.9 to 7.0 between 2007 and 2010.

Chironomus sp. NAIII (possibly *C. decumbens* (Malloch 1934))

Material examined (Table S1): 52 larvae collected in Lake D'Alembert (Quebec) and Hannah Lake, McFarlane Lake, Raft Lake and Ramsey Lake (Ontario).

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both the *cox1* and *gb2β* genes. *C*. sp. NAIII sequences form a distinct clade in both the *cox1* and *gb2β* ID-trees. Like *C. cucini*, the *C*. sp. NAIII *gb2β* sequence contains 3 extra base pairs at the 3' end, but unlike *C. cucini*, whose *gb2β* sequence has no intron, the *C*. sp. NAIII *gb2β* sequence contains a type II intron. Additionally, unlike the other *Chironomus* species (except *C. 'tigris'*) the *gb2β* of *C*. sp. NAIII is three codons short immediately after the end of the 2nd intron. *Cox1* PCR-RFLP analysis correctly separated *C.* sp. NAIII from the other *Chironomus* species. Sequences of *C*. sp. NAIII did not cluster with any of the available *Chironomus* species reference sequences.

Morphology (Table 6). Our specimens are medium sized salinarius-type larvae that are difficult to distinguish from those of *C. cucini* (see comments on the separation of these species under *C. cucini*). The morphology of *C*. sp. NAIII (see Table 6) is similar to that of *C. decumbens* (see Martin 2013).

Cytology. This species has three polytene chromosomes with heterochromatic centromeres. The arm combination is modified thummi-complex AB, CD, GEF. A nucleolus is located near the junction of arm G with arm E and a Balbiani ring is located towards the other end of arm G. Cytologically, this species fits the description of the North American cytospecies *C*. sp. 2x (Martin 2013) from Alaska, which is thought to be *C. decumbens* (Jim Sublette, personal communication). The only difference between our specimens and *C*. sp. 2x, is that our larvae possess a heavily heterochromatic centromere. This difference may or may not be significant since the presence of a heavily heterochromatic centromere can differ between populations and its detection can vary with the stain used. The voucher *C*. sp. 2x slide was stained with a brand of orcein that gave much paler staining.

Unfortunately, no *cox1* or *gb2β* sequences of *C. decumbens* voucher specimens were available for comparison with sequences for our study larvae. Further investigation on a possible relationship between *C. decumbens* and *C*. sp. NAIII is clearly warranted.

Distribution and ecology (Table 7). This species was found in oligotrophic to mesotrophic lakes at depths varying from 5–12 m and at pHs varying from 7.1–7.9.

Chironomus sp. NAI (*C. anthracinus*-group)

Material examined (Table S1): 9 larvae from Kasten Lake (Ontario).

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both the *cox1* and *gb2β* genes. This species forms a distinct clade in the *cox1* ID-tree. However in the *gb2β* ID-tree, sequences of *C*. sp. NAI cluster with those of *C. anthracinus*. Chosen restriction enzymes for the *cox1* PCR-RFLP analysis correctly separated *C.* sp. NAI from the other *Chironomus* species. Available DNA barcodes did not allow us to identify *C.* sp. NAI (see discussion at the end of this section).

Morphology (Table 6). Our specimens are large sized thummi-type larvae with the anterior ventral tubules slightly longer than the posterior ones. Morphologically, larvae of *C.* sp. NAI and *C. anthracinus* are indistinguishable.

Cytology. *C.* sp. NAI has four short polytene chromosomes with the thummi arm combination of AB, CD, EF, G. The most common sequence in each chromosome arm is similar to that in *C. anthracinus.*

Distribution and ecology (Table 7). This species was found in an oligotrophic, circum-neutral (pH 6.8), lake at a depth of 7.5 m.

Taxonomic comment. *C.* sp. NAI larval morphology and cytology strongly resemble those of *C. anthracinus*. Additionally, *gb2β* sequences of *C*. sp. NAI and *C. anthracinus* cluster together in the ID-tree (sequence divergence varies from 1 to 5%). In fact, there are no consistent base differences between these two *gb2β* sequences. Despite this lack of morphological, cytological or genetic difference, *cox1* sequences suggest that *C*. sp. NAI is a distinct species. The *cox1* sequence divergence between *C*. sp. NAI and *C. anthracinus* is relatively high (4–6%), and these sequences consistently differ by 22 bases (Table S4). For comparative purposes, other interspecific differences can be much lower, with *C. staegeri* and *C. 'tigris'* differing by only 9 specific bases, *C. frommeri* and *C. 'tigris'* by 13 specific bases, and *C. staegeri* and *C. frommeri* by specific 19 bases.

In the *cox1* ID-tree, the reference sequence for Palearctic *C. anthracinus* larvae from Lake Esrom (Denmark) clusters with larvae from our lakes that have been cytologically identified as *C. anthracinus*, not with the adjacent cluster formed by our *C*. sp. NAI sequences. We hypothesize that larvae of *C.* sp. NAI might be *C. rempelii* Thienemannn (1941). Currently, there are conflicting opinions as to the status of *C. rempelii*. Based on adult morphology, Townes (1945) concluded that *C. rempelii* was a synonym of *C. anthracinus* Zetterstedt (1860). Shobanov *et al.* (1996) and Kiknadze *et al.* (2005) reached the same conclusion when they compared the chromosomes of these species. However, the heterochromatin on arm F and the sequences A3, C3 and F3 have so far been found only in samples from western Canada that include the type locality of *C. rempelii* (British Columbia, Alberta, Saskatchewan and Manitoba; Kiknadze *et al.* 2005). The large hetrochromatic block in the original *C. rempelii* population occurred in all males. However, no other populations were sexed, and the smaller blocks and the inversions are rare. Consequently, the absence of these in our material is not conclusive. Samples from the type locality of *C. rempelii* would be required to confirm our hypothesis. We amplified the *cox1* sequence of a *Chironomus* larva from British Columbia (Marion Lake) that was morphologically and cytologically indistinguishable from that of *C. anthracinus* (labelled as "C. (*anthracinus*-group.)" in Fig. 8) and found that its *cox1* sequence (Fig. 8) clusters with sequences of *C.* sp. NAI. Thus *cox1* sequences suggest that the currently recognized C. *anthracinus* in North America is not a single species, but a complex of at least two closely related species—the *C. anthracinus*-group. This may be the result of a recent speciation that occurred without hybridization, so that *cox1* has differentiated while the short and slower evolving *gb2β* sequence has not had time to accumulate significant changes. Further investigations of *C.* sp. NAI and the species status of *C. rempelii* are clearly warranted.

Chironomus (*Chironomus*) *anthracinus* Zetterstedt (1860)

Material examined (Table S1): 27 larvae from Hannah Lake, Pine Lake, Raft Lake, Ramsey Lake and Silver Lake in Ontario and from Lake Arnoux and Lake Osisko in Quebec.

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both the *cox1* and *gb2β* genes. *C. anthracinus* sequences form a distinct clade in the *cox1* ID-tree and cluster with the Palearctic *C. anthracinus* reference sequence, which confirms the identification of this species. In the *gb2β* ID-tree, *C. anthracinus* sequences cluster with sequences of *C*. sp. NAI, the identity of which is uncertain (see discussion in the *C*. sp. NAI section). Chosen restriction enzymes for the *cox1* PCR-RFLP analysis correctly separated *C. anthracinus* larvae from the other *Chironomus* species.

Morphology. Our specimens are large sized thummi-type larvae whose anterior ventral tubules are usually longer than their posterior ones. Morphologically, larvae of *C. anthracinus* and *C.* sp. NAI are identical.

Cytology. The cytology of the 5 larvae analyzed was consistent with *C. anthracinus*, but identical to *C*. sp. NAI. *C. anthracinus* has 4 relatively short chromosomes with the thummi arm combination of AB, CD, EF, G (Kiknadze *et al.* 2005). The short chromosomes mean that banding patterns are often difficult to see clearly. There are two nucleoli: one on arm G and the other on arm F.

Distribution and ecology (Table 7). *C. anthracinus* is widely distributed in the Holarctic region. In the Nearctic region, it occurs across Canada (Alberta, British Colombia, Manitoba, Ontario and Saskatchewan) and the United States (California, Indiana, Massachusetts, New Hampshire New York and Wisconsin) (Sæther 1975; Oliver *et al.* 1990; Sæther 2012; Martin 2013). We found *C. anthracinus* at depths ranging from 2–12 m in highly acidic (pH 4.4) to circum-neutral (pH 8.5) lakes. The trophic status of these lakes was oligotrophic to mesotrophic, which is consistent with Sæther's (1975) suggestion that Nearctic *C. anthracinus* are more common in intermediate- to low-productivity lakes. In contrast, in the Palearctic region, *C. anthracinus* is known to be more frequently found in the profundal zone of moderately eutrophic lakes (Sæther 1975).

Chironomus (*Chironomus*) *entis* Shobanov (1989)

Material examined (Table S1): 8 larvae from Lake D'Alembert, Lake Dasserat, Lake Marlon, Lake Opasatica and Lake Pelletier (Quebec).

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both the *cox1* and the *gb2β* genes. In accordance with the findings of Makarevich *et al.* (2000), *C. entis gb2β* has a type II intron.

In the *cox1* ID-tree, sequences of cytologically identified *C. plumosus* and *C. entis* larvae cluster together. In fact, some *C. plumosus* and *C. entis* sequences are identical. Similarities between the mitochondrial nucleotide sequences of these two species were also observed by Guryev and Blinov (2002), who found that trees based on the mitochondrial *cytb* gene did not group populations of *C. entis* and *C. plumosus* according to their species affiliation but rather according to their geographic occurrence. They attributed this phenomenon to mitochondrial gene flow that occurs when populations of sympatric sibling species produce fertile hybrids such that mitochondrial DNA appears in the progeny of the backcross.

In contrast, in accordance with the findings of Guryev and Blinov (2002), our ID-tree based on nuclear *gb2β* gene sequences successfully groups *C. entis* and *C. plumosus* according to species. The intraspecific variability of the partial *gb2β* nucleotide sequences of our *C. entis* (0%) and *C. plumosus* (2.3%) larvae are considerably lower that their interspecific variability (15–16%). Thus *C. entis* and *C. plumosus* can be distinguished using the *gb2β* gene, but not the *cox1* gene. Lastly, since PCR-RFLP analysis was performed using the *cox1* gene, this result could not be used to separate *C. entis* and *C. plumosus*.

Morphology (Table 6). Our specimens are very large semireductus-type larvae. Morphologically, *C. entis* and *C. plumosus* are almost indistinguishable. Kiknadze *et al.* (1991) described the outer hooks on the anterior margin of the ventromental plates as being shorter and blunter in *C. plumosus* than in *C. entis* in Palearctic populations; however, we did not observe such differences in our specimens. *C. entis* are semireductus-type larvae, whereas those of *C. plumosus* vary from being semireductus-type to plumosus-type. In fact, some *C. plumosus* larvae from our study lakes have ventral tubules that are intermediate between the plumosus-type and semireductus-type. Within a given lake, the ventral tubules of *C. plumosus* were always longer than those of *C. entis*, which allowed the larvae of these species to be separated. However, between lakes, there was considerable overlap in their lengths. Consequently, *C. plumosus* and *C. entis* cannot be distinguished based solely on morphology such that cytological and genetic techniques are needed to unambiguously separate them.

Cytology. All larvae were analyzed cytologically to confirm the identification of this species and to verify the accuracy of the DNA results. *C. entis* has four relatively short chromosomes with the thummi arm combination of AB, CD, EF, G (Kiknadze *et al.* 2000a; Kiknadze *et al.* 2000b; Gunderina *et al.* 2009), with only a single nucleolus in arm G. It shares two rare sequences with Nearctic populations of *C. plumosus* (Kiknadze *et al.* 2000a).

Distribution and ecology (Table 7). *C. entis* has previously been reported from lakes in Canada (British Columbia, Manitoba, Ontario and Saskatchewan) and in the United States (Colorado, Indiana, Minnesota, North Dakota, Oklahoma, South Dakota and Wisconsin) (Kiknadze *et al.* 2000a; Martin 2013). We found *C. entis* in mesotrophic to eutrophic, circum-neutral (pH 7.1–8.3) lakes at depths ranging from 1–9 m.

Chironomus (*Chironomus*) *plumosus* Linnaeus (1758)

Material examined (Table S1): 33 larvae from lakes in Quebec (Lake D'Alembert, Lake Duprat, Lake Fortune, Lake Kinojévis, Lake Marlon, Lake Osisko, Lake Pelletier, Lake Rouyn and Lake Saint Augustin) as well as Kelly Lake in Ontario.

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both the *cox1* and *gb2β* genes. In accordance with the findings of Makarevich *et al.* (2000), *C. plumosus gb2β* has a type II intron. *C. plumosus* cannot be distinguished from *C. entis* on the basis of *cox1* sequences. However, these species can be separated using *gb2β* sequences (see comments in the *C. entis* DNA section).

Morphology (Table 6). Our specimens are very large semireductus (commonly referred to as "semi-reductus" type in reference to *C. plumosus*)—to plumosus-type larvae (see comment in section on *C. entis* morphology). Morphologically, larvae of *C. entis* and *C. plumosus* are indistinguishable (see comment in *C. entis* morphology section).

Cytology. All larvae were analyzed cytologically to confirm the identification of this species and to verify the accuracy of the DNA results. *C. plumosus* has four relatively short chromosomes with the thummi arm combination of AB, CD, EF, G (Butler *et al.* 1999) with only a single nucleolus in arm G.

Distribution and ecology (Table 7). This species was previously known from lakes in Canada (British

Columbia, Manitoba, Ontario and Saskatchewan) and the United States (Alabama, California, Colorado, Indiana, Kentucky, Massachusetts, Minnesota, New Mexico, North Dakota, Oklahoma, South Dakota and Wisconsin) (Butler *et al.* 1999; Martin 2013) at depths up to 23m (Martin 2013). We found *C. plumosus* at depths ranging from 1–8 m, and in oligotrophic to eutrophic lakes ranging in pH from 6.8–8.5.

Chironomus (*Chironomus*) *maturus* Johannsen (1908)

Material examined (Table S1): 10 larvae from Lake Bédard (Quebec).

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both the *cox1* and the *gb2β* genes. In both ID-trees, *C. maturus* sequences form a distinct clade. In the *cox1* ID-tree, sequences of the larvae we collected cluster with the reference sequence of *C. maturus*, thereby confirming the identification of this species. *Cox1* PCR-RFLP analysis accurately separated *C. maturus* larvae from the other *Chironomus* species.

Morphology (Table 6). Our specimens are large sized plumosus-type larvae with long ventral and lateral tubules. The frontoclypeus is pale; however, some *C. maturus* from other regions are reported to have a dark frontoclypeus (Martin 2013). Larvae of *C. maturus* could be distinguished from the other collected plumosus-type larvae through a combination of morphological features (see morphological key at the end of this section).

Cytology. *C. maturus* larvae were not identified through cytology because their chromosomes were not in good enough condition. *C. maturus* is known to possess four polytene chromosomes with a maturus arm combination of AF, BE, CD, G. The cytology of *C. maturus* has been described by Wülker and Martin (1974) and Kiknadze *et al.* (2004).

Description and ecology. This species has been recorded previously from shallow pools and polluted water bodies (Martin 2013) in central Canada (Manitoba and Ontario) and the United States (Alaska, California, Dakota, Indiana, Louisiana, New Mexico, New York, South Dakota and Wisconsin) (Oliver *et al.* 1990; Martin 2013). We collected *C. maturus* in a mesotrophic and circum-neutral (pH = 7.3) lake.

Chironomus (*Chironomus*) *decorus*-group sp. 2 Butler *et al.* (1995)

Material examined (Table S1): 17 larvae from Lake Adéline, Lake Dufault, Lake Duprat, Lake Fortune, Lake Opasatica and the St. Charles River in Quebec as well as Silver Lake in Ontario.

DNA (Figs. 8–9 and Tables 4–5, S3). In the *cox1* ID-tree, *C. decorus*-group sp. 2 nucleotide sequences form a distinct clade. Sequences of larvae we collected cluster with the reference sequence of *C. decorus*-group sp. 2 from GenBank, therefore confirming the identity of this species. The *cox1* interspecific sequence divergence between *C. decorus*-group sp. 2 and reference sequence *C. quinnitukqut* (3%) (data not shown) is within the intraspecific sequence divergence range (0–3%) of *Chironomus* species assessed in this study. Therefore, sequence divergence cannot be used to separate these species. *Cox1* PCR-RFLP analysis correctly separates *C. decorus*-group sp. 2 from the other *Chironomus* species. We were not successful in amplifying the *gb2β* gene of this species.

Morphology (Table 6). Our specimens are mostly medium sized bathophilus- or fluviatilis-type larvae (Table S1). However, whereas Quebec specimens had no lateral tubules, those from Silver Lake, Ontario, had small lateral tubules (melanotus-type). The 3rd inner tooth of the mandibles is pale and fused to the lower margin, however, in lakes from other regions, they are reported to be partially darkened (Martin 2013). From the morphological description of *C. quinnitukqut* in Martin (2013), larvae of *C. decorus*-group sp. 2 that do not possess lateral tubules could not be distinguished from those of *C. quinnitukqut*. Furthermore, larvae of *C. decorus*-group sp. 2 cannot be distinguished from those of *C. bifurcatus*.

Cytology. The cytology of the two larvae analyzed clearly indicates that they belong to a species currently referred to as *C. decorus*-group sp. 2. *C. decorus*-group sp. 2 has four polytene chromosomes with the thummi arm combination of AB, CD, EF, G (Butler *et al.* 1995). Typical of members of the *C. decorus*-group, it has only a single nucleolus, which is virtually terminal in arm G.

Description and ecology. This species has been collected previously in Canada (Saskatchewan) and the United States (Massachusetts, Minnesota, Mississippi, New Mexico, North Dakota, Vermont and Wisconsin) (Butler *et al.* 1995; Martin 2013). *C. decorus*-group sp. 2 larvae are reported from depths greater than 10 m (Martin

2013); however, in our study *C. decorus*-group sp. 2 larvae were found at depths of 1–5 m. *C. decorus*-group sp. 2 was collected in oligotrophic to mesotrophic lakes of circum-neutral pH (7.2–7.6). However, it was also found in the St. Charles River where sediments have been contaminated by untreated municipal waste waters.

Chironomus (*Chironomus*) *harpi* Sublette (in Wülker *et al.* 1991)

Material examined (Table S1): 6 larvae from Lake Arnoux (Quebec).

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both the *cox1* and *gb2β* genes. *C. harpi* sequences form distinct clades in both the *cox1* and *gb2β* ID-trees. *Cox1* sequences of collected larvae cluster with the reference sequence of *C. harpi*, thus confirming the identification of this species. *Cox1* PCR-RFLP profiles were not obtained for *C. harpi*. However, through simulation digests, we were able to demonstrate that the chosen restriction enzymes (*SspI*, *HinfI*, *RsaI* and *TaqI*) would have accurately separated *C. harpi* from the other *Chironomus* species.

Morphology (Table 6). Our specimens fit the morphological description of *C. harpi* (Martin 2013). They are medium sized plumosus-type larvae with posterior ventral tubules longer than the anterior ones. Morphologically, larvae of *C. harpi* strongly resemble those of the other plumosus-type larvae collected in our study by having a pale frontoclypeus (*C. frommeri*, *C. staegeri* and *C. maturus*). *C. harpi* larvae can be distinguished from larvae of these other species principally by the type of the middle trifid tooth on the mentum (see Table 6).

Cytology. We did not identify *C. harpi* through cytology. They are reported to possess four polytene chromosomes with the thummi arm combination of AB, CD, EF, G, with a large nucleolus near the centromere of arm D, and a second nucleolus sometimes developed medially in arm G. (Wülker *et al.* 1991).

Distribution and ecology (Table 7). In our study, we found *C. harpi* at depths of 1–4 m in a lake that has been heavily impacted by acid mine drainage (pH 2.7–3.8). This further supports the identification of this species, since *C. harpi* had been reported previously only from acidic pools (Arkansas, Illinois, New York and South Dakota) (Martin 2013).

Chironomus (*Chironomus) bifurcatus* Wuelker, Martin, Kiknadze, Sublette and Michiels (2009)

Material examined (Table S1): 50 larvae collected in Quebec (Lake Adéline, Lake Arnoux, Lake D'Alembert, Lake Duprat, Lake Kinojévis, Lake Opasatica and Lake St. Joseph) and in Ontario (McFarlane Lake and Silver Lake).

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both the *cox1* and *gb2β* genes. In the *cox1* ID-tree, sequences of collected larvae cluster with the reference sequences of *C. bifurcatus*, thereby confirming the identification of this species. However, in the *cox1* ID-tree, collected and reference *C. bifurcatus* nucleotide sequences form two distinct clades (labelled in Fig. 8 as group 1 and group 2) that differed by 7 specific bases (Table S5). There were also consistent cytological differences between these two groups (see cytology section). However, the sequence divergence between *cox1*-sequences from these two groups is low (2%) and, in the *gb2β* ID-tree, *C. bifurcatus* sequences from both groups cluster together.

Three different profiles were created through PCR-RFLP analysis of the *cox1* gene. One profile included all group 1 larvae and the other two profiles (obtained using the *TaqI* enzyme) included all group 2 larvae. Simulation digests demonstrate that the *SspI*, *HinfI*, *RsaI* and *TaqI* restriction enzymes would not have separated *C. bifurcatus* (gr. 2) larvae from *C. dilutus* because in some cases their restriction fragment lengths are so similar that it would be difficult to differentiate them on an agarose gel. However, simulation digests demonstrate that both species should be separable using the restriction enzyme *AluI*.

Morphology (Table 6). Our specimens are medium sized bathophilus-type larvae with the anterior ventral tubules slightly longer than the posterior ones. All of our larvae lacked lateral tubules. In contrast, larvae from some populations of *C. bifurcatus* are reported to have either very small (180 µm) lateral tubules (about 180 µm; Wuelker *et al.* 2009) (melanotus-type) or be fluviatilis-type (Martin 2013). The frontoclypeus of our *C. bifurcatus* larvae is pale, but is reported to be slightly darkened in larvae from some other regions (Wuelker *et al.* 2009). There were no morphological differences between larvae belonging to groups 1 and 2. Morphologically, larvae of *C. bifurcatus* cannot be distinguished from those of *C. decorus*-group sp. 2.

Cytology. The cytology of the 5 specimens analyzed permitted us to identify them as *C. bifurcatus*. Larvae of this species have four polytene chromosomes with the thummi arm combination of AB, CD, EF, G, as well as a single, virtually terminal, nucleolus in arm G (Wuelker *et al.* 2009). However, we noted some cytological differences among our larvae and among reference specimens that allowed all of the specimens to be separated into two groups that corresponded to those mentioned above for the *cox1* gene. Thus larvae from group 1 have cytological sequences B1 and F1 with no median Balbiani ring in the middle of arm G, whereas those from group 2 have cytological sequences B2 and F2, and a Balbiani ring in the middle of arm G.

Distribution and ecology (Table 7). This species has been collected previously in southern Canada (Manitoba, Ontario and Quebec) and the northern United States (Massachusetts, Michigan, Minnesota and Wisconsin) (Wuelker *et al.* 2009; Martin 2013). We collected larvae of *C. bifurcatus* gr. 1 only in Quebec lakes (Lake Arnoux, Lake D'Alembert, Lake Duprat, Lake Opasatica and Lake St. Joseph), whereas those in gr. 2 were found in lakes in both Quebec (Lake Kinojévis and Lake St. Joseph) and Ontario (McFarlane Lake and Silver Lake). Larvae belonging to the two genetic groups differed somewhat in their distribution with respect to lake water pH, trophic status and water depth. Specifically, larvae of *C. bifurcatus* (gr. 1) were collected in acidic to circum-neutral (pH 2.7–7.6) and oligotrophic to eutrophic lakes at depths ranging from 1.5–9 m. In contrast, larvae of *C. bifurcatus* (gr. 2) were collected in circum-neutral (pH 7.0–7.8) and oligotrophic to mesotrophic lakes over a wider range of depths (4–24m). We note that larvae of *C. bifurcatus* (gr. 2) were always found along with those of *C. staegeri* and *C. 'tigris'* whereas this was not the case for gr. 1 larvae.

Taxonomic comments. Although *cox1* sequences and cytological differences separate *C. bifurcatus* into two groups, we suggest that it is premature to recognize two species until further work has been completed.

Chironomus (*Chironomus*) *dilutus* Shobanov, Kiknadze and Butler (1999)

Material examined (Table S1): 9 larvae from Kelly Lake (Ontario).

DNA (Figs. 8–9 and Tables 4–5, S3). We were able to obtain PCR products for both *cox1* and *gb2β* genes. In accordance with the findings of Makarevich *et al.* (2000), *C. dilutus gb2β* has no intron. In the *cox1* ID-tree (Fig. 8), reference sequences of *C. pallidivittatus* (sensu Beermann 1955) cluster with reference sequences of *C. dilutus*. Similarities between the mitochondrial nucleotide sequences of these two species were also observed by Martin *et al.* (2002), who showed that trees based on mitochondrial sequences clustered populations of *C. dilutus* and *C. pallidivittatus* according to their geographic distribution, whereas those based on nuclear sequences clustered populations according to their species affiliation. The inability of the mitochondrial *cox1* gene to separate *C. dilutus* and *C. pallidivittatus* is likely due to mitochondrial gene flow (Martin *et al.* 2002). To confirm the identification of larvae whose *cox1* sequences clustered with reference *C. dilutus* and *C. pallidivittatus* sequences, we amplified and sequenced the partial *gb2β* gene. Partial *gb2β* gene sequences of all larvae were identical to the *C. dilutus* reference sequence (Fig. 9), which suggests that our larvae are not *C. pallidivittatus* but more likely *C. dilutus*. *Cox1* PCR-RFLP profiles were not obtained for *C. dilutus*. However, simulation digests demonstrated that the *SspI*, *HinfI*, *RsaI* and *TaqI* restriction enzymes would not have separated *C. dilutus* from *C. bifurcatus* and *C*. nr. *atroviridis* (sp. 2i) because their restriction fragment lengths are so similar in some cases that it would be difficult to differentiate them on an agarose gel. However, simulation digests demonstrate that *C. dilutus* could be distinguished from *C. bifurcatus* and *C*. nr. *atroviridis* (sp. 2i) using the restriction enzyme *AluI*.

Morphology (Table 6). Our specimens are large sized plumosus-type larvae with their posterior ventral tubules usually longer than their anterior ventral tubules. Although the teeth of the mentum and mandibles of *C. dilutus* are reported to be rounded (Martin 2013), in our larvae, they varied from being rounded to sharp, which suggests that this feature cannot be used to identify larvae of *C. dilutus* in our study area. Morphologically, larvae of *C. dilutus* cannot be distinguished from those of *C. pallidivittatus* (not collected in our study; see Martin 2013).

Cytology. *C. dilutus* larvae have four polytene chromosomes with the camptochironomus arm combination of AB, DE, CF, G. The cytology of *C. dilutus* has been described by several authors (see Martin 2013).

Distribution and ecology (Table 7). This species has been found previously in numerous localities across Canada (Alberta, British Columbia, Manitoba, Ontario and Saskatchewan) and the northern United States (Iowa, Massachusetts, Michigan, Minnesota, New York, North Dakota, South Dakota, Utah, Wisconsin and Wyoming) (Shobanov *et al.* 1999; Martin 2013). *C. dilutus* is known to thrive in organically-enriched eutrophic water bodies (Townes 1945, referred to as *Tendipes (Tendipes) tentans* (Fabricius)). We collected large numbers of *C. dilutus* in

eutrophic Kelly Lake (at 5 m, pH 7.5) where sediments have been highly contaminated by untreated sewage from the city of Sudbury (1880s–1972) along with discharges from mining, milling and smelting operations (1880s–present) (City of Greater Sudbury 2013). Most of the larvae we collected had deformed mouthparts, likely due to the contaminants to which they were exposed (Hare & Carter 1976).

Chironomus (*Chaetolabis*) *ochreatus* Townes (1945)

Material examined (Table S1): 2 larvae from Lake Opasatica (Quebec).

DNA (Figs. 8–9 and Tables 4–5, S3). In the *cox1* ID-tree, *C. ochreatus* nucleotide sequences clearly form a distinct clade. Sequences of collected species cluster with the reference sequence of *C. ochreatus* thereby confirming the identification of this species. We were not successful in amplifying the *gb2β* gene for this species and *cox1* PCR-RFLP profiles were not obtained for this species. However, simulation digests using the restriction endonucleases *Ssp*I, *Hinf*I, *Rsa*I and *Taq*I demonstrate that *cox1* PCR-RFLP analysis would have separated *C. ochreatus* from the other *Chironomus* species.

Morphology (Table 6). Our specimens are medium sized thummi-type larvae. The 3rd inner teeth of the mandibles are partially darkened and fused to the lower margin (type B; Fig. 6). In contrast, in specimens studied by Martin (2013), the 3rd inner tooth was pale and well separated from the lower margin.

Cytology. The cytology of the two larvae analyzed clearly indicates that this species is *C. ochreatus*. *C. ochreatus* has three polytene chromosomes that are thought to have a modified thummi arm combination of AB, CD, GEF (Martin 2012). Arm G is generally unpaired with a nucleolus near the junction with arm E.

Distribution and ecology (Table 7). This species was previously known from the eastern United States (Arkansas, Maine, Massachusetts, Michigan, New Jersey, New York, Rhode Island, South Carolina, Virginia and Wisconsin) (Townes 1945; Oliver *et al.* 1990; Martin 2013). In our study, we collected *C. ochreatus* in a single mesotrophic Quebec lake at a depth of 2 m (pH 7.7).

Chironomus (*Chaetolabis*) nr. *atroviridis* (sp. 2i) Martin (2013)

C. atroviridis Townes has been found to comprise two species in North America, one with four polytene chromosomes (sp. 2i of Martin 2013), and the other with only three polytene chromosomes (sp. 2h of Martin 2013). Only the former species occurred in our samples.

Material examined (Table S1): 4 larvae from Lake Marlon (Quebec).

DNA (Figs. 8–9 and Tables 4–5, S3). In the *cox1* ID-tree, *C.* nr. *atroviridis* (sp. 2i) nucleotide sequences form a distinct clade. Sequences of collected larvae cluster with the reference sequence of *C*. nr. *atroviridis* (sp. 2i), which confirms the identification of this species. We were not successful in amplifying the *gb2β* gene of this species. *Cox1* PCR-RFLP profiles were not obtained for this species. However, simulation digests demonstrate that *Ssp*I, *Hinf*I, *Rsa*I and *Taq*I could not be used to separate *C*. nr. *atroviridis* (sp. 2i) from all *C. dilutus* specimens because their restriction fragment lengths are so similar that it would be difficult to differentiate them on an agarose gel. However, simulation digests demonstrate that both species could be separated using the restriction enzyme *Alu*I.

Morphology (Table 6). Our specimens are large sized thummi-type larvae. They are morphologically very similar to those of *C. ochreatus*, in that both species have the teeth of the pecten epipharyngis flattened, however, the gula of *C.* nr. *atroviridis* (sp. 2i) is darkened posteriorly whereas that of *C. ochreatus* is pale or only slightly darkened.

Cytology. The cytology of the 2 larvae analyzed clearly indicates that this species is *C*. nr. *atroviridis* (sp. 2i). *C.* nr. *atroviridis* (sp. 2i) has four polytene chromosomes with some indication of a thummi arm combination (Martin 2013). Arm G is generally unpaired with a virtually terminal nucleolus. There are no nucleoli in the other chromosomes.

Distribution and ecology (Table 7). This species has been collected previously in Manitoba and Ontario in shallow water near macrophytes (Martin 2013). Likewise, we collected *C.* nr. *atroviridis* (sp. 2i) in the vegetated littoral zone (1 m depth) of a mesotrophic to eutrophic lake (pH 7.4).

Taxonomic comment. Wiederholm (1979) considered *C. ochreatus* to be a synonym of *C*. *atroviridis*, but

mentioned that further study was needed. Because Wiederholm (1979) was not aware that there were two forms of *C. atroviridis* (2i and 2h; Martin 2013), we do not know which form would correspond to the material he was comparing. In any case, our results clearly indicate that *C.* nr. *atroviridis* (sp. 2i) is distinct from *C. ochreatus*. First, the *cox1* sequences of *C.* nr. *atroviridis* (sp. 2i) and of *C. ochreatus* are strikingly different such that their average interspecific divergence (12%; Table 5) is much higher than their intraspecific divergences (<3%; Table S3). Second, *C.* nr. *atroviridis* (sp. 2i) has 4 chromosomes whereas *C. ochreatus* has only 3. *C. ochreatus* also differs from the three chromosome forms of *C.* nr. *atroviridis* (sp. 2h) by the position of the nucleolus, which is subterminal in the latter species.

Morphological key to larvae of the *Chironomus* species collected in our study

The following key for identifying fourth-instar larvae of the *Chironomus* species collected in our study is based on the morphology of the tubules on the 10th and 11th body segments and features of the head capsule. Illustrations of these features are given in Figures 1–7.

Although the majority of our study species can be separated using the following key, we acknowledge that, as with most morphological classifications, the characters used are likely to show some variability due to genetic or environmental factors. In this key, larvae are first separated based on the absence/presence, length and shape of tubules, which was effective in separating larvae of all of our collected species with the exception of *C. decorus*-group sp. 2 since some larvae of this species had lateral tubules whereas others did not. The ventral tubules of *C. decorus*-group sp. 2 larvae also varied from being straight (bathophilus-type) to slightly curved (fluviatilis-type). Our *C. bifurcatus* larvae were all bathophilus-type, but in other geographical regions have been ascribed to several larval types (bathophilus, fluviatilis or melanotus; Martin 2013) and these differences are reported to be related to the depth or the type of substrate on which larvae occur (Martin 2013). At an extreme, the presence or absence of lateral tubules among *C. bifurcatus* larvae is reported to vary among larvae hatched from the single egg mass from which the type was reared. (J. Martin, unpublished).

Note that this key is based on morphological features of the *Chironomus* species that we collected, such that other species in the Nearctic could fit these descriptions (see notes at the end of the key).

1	11th segment without ventral tubules; 10th segment without lateral tubules (salinarius-type larvae)	2
-	11th segment with one or two pairs of ventral tubules	3
2	Frontoclypeus with a dark longitudinal stripe and a lobed dark spot in the middle; central trifid tooth of mentum with outer teeth almost completely separated from middle tooth (type C); 3rd inner tooth of mandibles partially darkened and fused to lower margin (type B)	*C.* sp. NAII [1]
-	Frontoclypeus pale or slightly darkened with a lobed dark spot in anterior portion; central trifid tooth of mentum with outer teeth partially separated from middle tooth (type B); 3rd inner tooth of mandibles pale and fused to lower margin (type A)	*C. cucini* (in part) or *C.* sp. NAIII [1 and 2]
3	11th segment with only one pair of short ventral tubules (located posteriorly); 10th segment without lateral tubules (halophilus-type)	*C. cucini* (in part)
-	11th segment with two pairs of ventral tubules	4
4	10th segment without lateral tubules	5
-	10th segment with a pair of lateral tubules	8
5	Ventral tubules straight (bathophilus-type) or slightly curved (fluviatilis-type)	*C. bifurcatus* (in part) or *C. decorus*-group sp. 2 (in part) [3]
-	Anterior ventral tubules with an elbow; posterior ventral tubules coiled (thummi-type)	6
6	Pecten epipharyngis teeth flattened (type C or D)	7
-	Pecten epipharyngis teeth elongated (type A or B)	*C. anthracinus* or *C.* sp. NAI
7	Gular region almost completely pale or at most slightly darkened	*C. ochreatus*
-	Gular region darkened posteriorly	*C.* nr. *atroviridis* (2i)
8	Ventral tubules straight or slightly curved	9
-	Anterior pair of ventral tubules with an elbow; posterior pair of ventral tubules coiled (plumosus-type larvae)	10
9	Ventral tubules equal to or greater than the width of the 11th segment (melanotus type); 3rd inner tooth of mandible pale	*C. decorus*-group sp. 2 (in part) or *C. bifurcatus* (in part)
-	Ventral tubules less than the width of the 11th segment (semireductus-type larvae); 3rd inner tooth of mandible dark	*C. entis* or *C. plumosus* (in part)
10	3rd inner tooth of mandible partially dark to dark	11
-	3rd inner tooth of mandible pale	12

11	Frontoclypeus pale and gula strongly to completely darkened	*C. plumosus* (in part)
-	Frontoclypeus dark and gula slightly to posteriorly darkened	*C. dilutus* [4]
12	Anterior margin of ventromental plates crenulated	*C. frommeri* or *C. staegeri* [5]
-	Anterior margin of ventromental plates smooth to relatively smooth	13
13	Frontoclypeus pale	14
-	Frontoclypeus dark	15
14	3rd inner tooth of mandible fused to lower margin, central trifid tooth of mentum with outer teeth only partially separated from middle tooth (type B) and 4th lateral teeth reduced to the height of the 5th lateral teeth (type II); found in highly acidic waters	*C. harpi*
-	3rd inner tooth of mandible separated from lower margin, central trifid tooth of mentum with outer teeth distinctly separated from middle tooth (type D) and 4th lateral teeth only slightly reduced (type I)	*C. maturus* (in part)
15	Gula pale to slightly darkened and 4th lateral teeth of mentum only slightly reduced (type I)	*C. maturus* (in part)
-	Gula strongly to completely darkened and 4th lateral teeth of mentum about the same height as the 5th lateral teeth (type II)	*C. 'tigris'*

Notes:

[1] It is likely that *C. atritibia* (Malloch 1934) would also key here, as it is reported to have a salinarius-type larva (Wülker & Butler 1983). Although *C. atritibia* is thought to have a more northerly distribution, we cannot rule out the possibility that it corresponds to either *C.* sp. NAII or *C*. sp. NAIII.

[2] In our specimens, the mean AR of *C. cucini* (2.08) was significantly greater than that of *C.* sp. NAIII (1.77). Although there was some overlap in the range of ARs between the two species, 4 of 5 *C. cucini* larvae had an AR >2.0, whereas 10 of 11 specimens of *C.* sp. NAIII had an AR <1.95. The more southern *C. major* (Wülker & Butler 1983) would also key here, but is much larger (30–55 mm; Epler 2001).

[3] Other Nearctic species of the *C. decorus*-group (Wuelker 2010; Sæther 2012) are also likely to key out here (ex. *C. quinnitukqut*)

[4] *C. pallidivittatus* would also key out here.

[5] *C. crassicaudatus* (Malloch 1915) would also key out here.

Approaches used to delimit *Chironomus* species

No single approach (morphology, cytology, genetics) was adequate for delimiting and identifying larvae of all of the *Chironomus* species that we collected.

Thus larval morphology alone could not be used to separate five pairs of *Chironomus* species that we collected, i.e., *C. cucini* and *C*. sp. NAIII, *C. bifurcatus* and *C. decorus*-group sp. 2, *C. anthracinus* and *C*. sp. NAI, *C. staegeri* and *C. frommeri*, as well as *C. entis* and *C. plumosus*. Furthermore, some Nearctic species that we did not collect are reported to be morphologically identical to our study species (see notes at the end of the morphological key). In addition, for some of our species, specimens from other regions are reported to differ morphologically from those that we collected, and it is known that some larval characters are affected by wear, environmental conditions and genetic variation (Martin 2013). This said, larval morphology was undeniably important when used in combination with other methods of species delimitation.

Differences in larval cytology, based on the structure of salivary-gland polytene chromosomes, allowed the definitive identification of many of the species that we collected. Indeed, the cytology of most North American *Chironomus* species has been described (Martin 2013). However, one must bear in mind that cytology is faced with the same challenges as the other identification methods; that is, it is not always possible to determine whether or not differences in chromosome banding patterns and other structures are attributable to species differences or to regional or individual differences within a given species (Martin 2011). Indeed, only a handful of taxonomists worldwide have the necessary expertise to identify *Chironomus* species through cytology, which is a major drawback for non-cytological experts wishing to identify *Chironomus* species.

Genetic techniques, namely PCR-RFLP analysis and DNA barcoding of the *cox1* gene, successfully separated and identified most of the *Chironomus* species that we collected. We present the first *cox1* sequences for many of the known Nearctic *Chironomus* species (*C. acidophilus*, C. *anthracinus*, *C. bifurcatus*, *C. cucini*, *C. frommeri*, *C. harpi*, *C.* nr. *atroviridis* (sp. 2i), *C. ochreatus*, *C. plumosus*, *C. quinnitukqut*, *C.* sp. g, *C*. sp. h and *C. 'tigris'*). However, DNA barcoding failed to distinguish between two species pairs (*C. entis* and *C. plumosus*; *C. dilutus* and *C. pallidivittatus*) because each pair has identical *cox1* nucleotides sequences. Such sequence similarities are likely the result of mitochondrial gene flow, and have been found in a number of closely related species groups around the world (e.g. Martin 2011).

Using the *gb2β* gene, we successfully separated *C. entis* from *C. plumosus* and *C. dilutus* from *C. pallidivittatus* and confirmed the species statuses of *C. cucini*, *C. bifurcatus*, *C. harpi*, *C. maturus*, *C*. sp. NAIII and *C. 'tigris'*. We present the first published *gb2β* sequences for *C. anthracinus*, *C. bifurcatus*, *C. calligraphus*, *C. cucini*, *C. harpi*, *C. maturus*, *C*. sp. u and *C. 'tigris'*. One downside of using the *gb2β* gene was that we were not able to obtain PCR products for all species. In fact, no primer combination was able to amplify all *Chironomus* species, which is a limitation when using this gene for DNA barcoding. For these species we sometimes obtained two PCR products or sequences belonging to the *globin 7A* or *9* genes. Our results confirm those of Hankeln *et al.* (1997) who found that this gene is highly variable and that the only conserved regions are also conserved in the *gb7A* and *gb9* genes. This is a major drawback for using the *gb2β* gene for identifying *Chironomus* species. Thus, the use of another nuclear gene might be more appropriate for *Chironomus* species identifications. Other studies have used the nuclear internal transcribed spacer (ITS) region to separate *C. plumosus* from *C. entis* as well as other *Chironomus* species (Gunderina & Katokhin 2011; Martin 2011; Gunderina 2012). The nuclear *carbamoylphosphate synthetase* (CAD) region has also been successfully used to identify chironomid species (Carew *et al.* 2011). Another disadvantage of using the *gb2β* gene is that nuclear genes evolve more slowly than do mitochondrial genes. Thus, relatively recent speciation might not always be detected when using the *gb2β* gene. This might be the reason why sequences of *C*. sp. NAI and *C. anthracinus*, as well as those of *C. bifurcatus* (gr. 1) and *C. bifurcatus* (gr. 2), differ for the *cox1* gene but are identical for the *gb2β* gene. Thus, incorporation of both mitochondrial and nuclear genes, whose modes of inheritance and mutation rate differ, clearly provides better resolution for *Chironomus* species identification.

Several studies have advocated the use of sequence divergence thresholds to separate species (ex. Hebert *et al.* 2004b). However, our results demonstrate that sequence divergence thresholds cannot be used to separate all *Chironomus* species. Thus we recorded overlap between intra- and inter-specific sequence divergences for both of the genes that we studied (Table 5); similar overlaps have been reported for other *Chironomus* species (Martin 2011), as well as for species of other chironomid genera (Carew *et al.* 2005; Ekrem *et al.* 2007) and other types of dipterans (Meier *et al.* 2006). In our study, *Chironomus cox1* intraspecific sequence divergences were $\leq$ 3% (Table S3). For most species, *cox1* interspecific sequence divergences ranged from 9% to 20%, but between some of our study species the interspecific divergences ranged from 1 to 4% (Table 5). This overlap was due either to some of our study species sharing identical sequences (i.e., *C. entis*/*C. plumosus* and *C. dilutus*/*C. pallidivittatus*) or to interspecific divergences being so low that they fell within the intraspecific range for the genus (i.e., *C. staegeri*/*C. 'tigris'*/*C. frommeri* (Table 5) and *C. decorus*-group sp. 2/*C. quinnitukqut* (data not shown)). With respect to the *gb2β* gene, *Chironomus* species intraspecific sequence divergences were $\leq$ 2%. The interspecific divergences between most species ranged from 5 to 46% (Table 5), but sequence divergences between *C*. sp. NAI and *C. anthracinus* ranged from 1 to 5% (Table 5), which is within the intraspecific range for species. In light of our results, the calculated intraspecific sequence divergences of 3% for the *cox1* gene and 2% for the *gb2β* gene can be used as a guide to help sort *Chironomus* species, but should not be used in isolation. DeSalle *et al.* (2005) have suggested that, rather than looking at sequence divergences, specific base differences that characterize related species should be sought. We used this approach to determine whether or not *C.* sp. NAI and *C. anthracinus*, as well as *C. bifurcatus* (gr. 1) and *C. bifurcatus* (gr. 2), are distinct species. However, even when using specific base differences, we are still faced with the same challenge inherent to other species-delimitating methods; that is, how much of a difference is needed for species to be considered different.

DNA barcoding is more precise than PCR-RFLP because it allows the exact determination of base pair differences between individuals. Nevertheless, when a large number of individuals need to be identified, PCR-RFLP has been advocated as a cost-effective technique to assess molecular variation (Pfrender *et al.* 2010). However, as the cost of sequencing continues to fall, sequencing is becoming the most effective and economical approach, even for determining large numbers of individuals. The disadvantage of the PCR-RFLP approach is that if the right enzymes are not chosen for analysis, sequence nucleotide differences can go unnoticed. In our study, the first chosen restriction enzymes (*Ssp*I, *Hinf*I, *Rsa*I and *Taq*I) were not able to discern differences in the *cox1* nucleotide sequences of *C. bifurcatus*, *C. dilutus* and *C*. nr. *atroviridis* (sp. 2i) as well as those of *C. staegeri*, *C. 'tigris'* and *C. frommeri*. Additional restriction enzymes were necessary to separate these species. Likewise, PCR-RFLP did not discriminate between *C. entis* and *C. plumosus* and it would not likely be able to separate *C. dilutus* from *C. pallidivittatus* because both of these species pairs share identical *cox1* sequences.

Overall, for non-cytological experts, we recommend the use of combined genetic and morphological

techniques to identify *Chironomus* larvae to species since this combination was much more effective than either of these techniques alone.

Overall conclusions and recommendations for identifying *Chironomus* species

Overall, using morphology, cytology and genetics we conclude that our 404 *Chironomus* larvae represent 17 species, 14 of which have been identified as *C*. (*Chaetolabis*) nr. *atroviridis* (sp. 2i), *C*. (*Chaetolabis*) *ochreatus*, *C*. (*Chironomus*) *anthracinus*, *C*. (*Chironomus*) *bifurcatus*, *C*. (*Chironomus*) *cucini*, *C*. (*Chironomus*) *decorus*-group sp. 2, *C*. (*Chironomus*) *dilutus*, *C*. (*Chironomus*) *entis*, *C*. (*Chironomus*) *frommeri*, *C*. (*Chironomus*) *harpi*, *C*. (*Chironomus*) *maturus*, *C*. (*Chironomus*) *plumosus*, *C*. (*Chironomus*) *staegeri* and *C*. (*Chironomus*) *'tigris'* while the identification of three others remains uncertain (*C*. sp. NAI-III). The species status of *C*. sp. NAI requires further investigation and additional studies are necessary to determine whether are not *C. bifurcatus* is a single species or a complex of at least two closely related species. Of the 14 identified *Chironomus* species, two belong to the subgenus *Chaetolabis* whereas 12 belong to the subgenus *Chironomus*.

We collected and identified 11 (*C. anthracinus*, *C. bifurcatus*, *C. cucini*, *C. decorus*-group sp. 2*, C. dilutus*, *C. entis*, *C. maturus*, *C*. nr. *atroviridis* (sp. 2i), *C. plumosus*, *C. staegeri* and *C. 'tigris'*) of the 20 *Chironomus* species currently known from the Canadian provinces east of the Rocky Mountains (that is, from Canada excluding British Columbia and the three northern territories; Martin 2013). Since all but three of the 31 water bodies that we sampled are located in the same ecozone (the Boreal Shield), some of the nine species that we did not find could be restricted to other ecozones, such as the prairies, where water chemistry and other factors are likely to differ from those in Boreal Shield lakes. In fact, it is surprising that we were able to collected so many *Chironomus* species from a single ecozone in which lake waters are generally nutrient poor, circum-neutral and soft (low concentrations of calcium and magnesium), which is likely to limit the range of habitats available for *Chironomus* species. This large proportion of known species is likely explained by the fact that our study lakes in this ecozone encompass wide ranges in these variables because some of them have been altered by discharges from mining, milling and smelting operations, or sewage treatment plants, or by the addition of lime to counter lake acidification (Lakes Arnoux, Osisko, Pelletier, Rouyn and Kelly).

The range of chemical conditions under which some of the *Chironomus* species were collected was quite wide. For example, *C. anthracinus* and *C. bifurcatus* were found in waters that were highly acidic to circum-neutral and *C. entis*, *C. bifurcatus*, C. *plumosus* and *C. staegeri* were found in water bodies that were oligotrophic to eutrophic. In contrast, *C. harpi* was restricted to a highly acidic lake and *C. dilutus* was collected only in a lake that had been organically enriched by sewage.

We found three *Chironomus* species (*C. frommer*i, *C. harpi* and *C. ochreatus*) that were previously known from the Nearctic (Martin 2013), but had not been reported from eastern Canada. The identification of another three species remains unclear (*C*. sp. NAI-III). We note that other species are likely to exist in eastern Canada, since 19 cytologically-defined but as yet unidentified or unassigned *Chironomus* species have been reported from this region (Martin 2013). Applying the combination of morphological and genetic techniques used in our study would likely resolve many of these taxonomic gaps in the Canadian and Nearctic fauna.

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TABLE S1. Individual *Chironomus* larvae indicating their associated: voucher code, location and year of collection, and species name. Performed *cox1* PCR-RFLP, morphological and cytological analyses are marked (x). For *cox1* and *gb2β* gene sequencing, GenBank accession numbers are given.

Voucher code	Larvae type	Locations	Year	Performed analysis					Species
				cox1 PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
AR10-TH1	thummi	AR	2010		KF278225	KF278426	x		*C. anthracinus*
AR10-TH2	thummi	AR	2010		KF278226	KF278427	x		*C. anthracinus*
HA07-TH1	thummi	HA	2007	x			x		*C. anthracinus*
HA07-TH2	thummi	HA	2007	x			x		*C. anthracinus*
HA07-TH3	thummi	HA	2007	x	KF278221		x		*C. anthracinus*
HA07-TH4	thummi	HA	2007	x			x		*C. anthracinus*
HA07-TH5	thummi	HA	2007	x			x		*C. anthracinus*
HA07-TH6	thummi	HA	2007	x			x		*C. anthracinus*
HA07-TH7	thummi	HA	2007	x			x		*C. anthracinus*
HA07-TH8	thummi	HA	2007	x	KF278222		x	x	*C. anthracinus*
HA07-TH9	thummi	HA	2007	x			x		*C. anthracinus*
HA07-TH10	thummi	HA	2007	x			x		*C. anthracinus*
OS10-TH1	thummi	OS	2010		KF278227	KF278393	x		*C. anthracinus*
OS10-TH2	thummi	OS	2010		KF278223		x		*C. anthracinus*
OS10-TH3	thummi	OS	2010		KF278228		x		*C. anthracinus*
PI10-TH1	thummi	PI	2010		KF278229	KF278395	x	x	*C. anthracinus*
PI10-TH2	thummi	PI	2010		KF278224		x		*C. anthracinus*
AnthRL21m	thummi	RA	2010		KF278232		x	x	*C. anthracinus*
RA10-TH1	thummi	RA	2010		KF278230	KF278394	x		*C. anthracinus*
RA10-TH4	thummi	RA	2010		KF278231	KF278425	x		*C. anthracinus*
RAM07-TH1	thummi	RM	2007	x			x		*C. anthracinus*
RAM07-TH2	thummi	RM	2007	x			x		*C. anthracinus*
RAM07-TH3	thummi	RM	2007	x	KF278233		x		*C. anthracinus*
RAM07-TH4	thummi	RM	2007	x			x	x	*C. anthracinus*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Year	Performed analysis					Species
				cox1 PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
RAM07-TH5	thummi	RM	2007	x			x		*C. anthracinus*
RAM07-TH6	thummi	RM	2007	x			x		*C. anthracinus*
SI11-TH1	thummi	SI	2011		KF278234		x		*C. anthracinus*
AD10-BA2	bathophilus	AD	2010		KF278316		x		*C. bifurcatus* (gr. 1)
AR10-BA1	bathophilus	AR	2010		KF278319	KF278435	x		*C. bifurcatus* (gr. 1)
AR10-BA2	bathophilus	AR	2010				x	x	*C. bifurcatus* (gr. 1)
AR10-BA3	bathophilus	AR	2010		KF278317	KF278436	x		*C. bifurcatus* (gr. 1)
AR10-BA4	bathophilus	AR	2010		KF278318		x		*C. bifurcatus* (gr. 1)
AL06-BA1	bathophilus	DA	2006	x	KF278315		x		*C. bifurcatus* (gr. 1)
AL06-BA2	bathophilus	DA	2006	x			x		*C. bifurcatus* (gr. 1)
AL06-BA3	bathophilus	DA	2006	x			x		*C. bifurcatus* (gr. 1)
AL06-BA4	bathophilus	DA	2006	x			x		*C. bifurcatus* (gr. 1)
AL06-BA5	bathophilus	DA	2006	x			x		*C. bifurcatus* (gr. 1)
AL06-BA6	bathophilus	DA	2006				x	x	*C. bifurcatus* (gr. 1)
DU06-BA1	bathophilus	DP	2006	x			x		*C. bifurcatus* (gr. 1)
DU06-BA2	bathophilus	DP	2006	x			x		*C. bifurcatus* (gr. 1)
DU06-BA3	bathophilus	DP	2006	x			x		*C. bifurcatus* (gr. 1)
DU06-BA4	bathophilus	DP	2006	x			x		*C. bifurcatus* (gr. 1)
DU06-BA5	bathophilus	DP	2006	x			x		*C. bifurcatus* (gr. 1)
DU07-BA1	bathophilus	DP	2007	x	KF278320		x		*C. bifurcatus* (gr. 1)
DU07-BA2	bathophilus	DP	2007	x			x		*C. bifurcatus* (gr. 1)
DU07-BA3	bathophilus	DP	2007	x			x		*C. bifurcatus* (gr. 1)
DU07-BA4	bathophilus	DP	2007	x			x		*C. bifurcatus* (gr. 1)
DU07-BA5	bathophilus	DP	2007	x			x	x	*C. bifurcatus* (gr. 1)
DU07-BA6	bathophilus	DP	2007	x			x		*C. bifurcatus* (gr. 1)
DU07-BA7	bathophilus	DP	2007	x			x		*C. bifurcatus* (gr. 1)

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Year	Performed analysis					Species
				cox1 PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
DU07-BA8	bathophilus	DP	2007	x			x		*C. bifurcatus* (gr. 1)
DU07-BA9	bathophilus	DP	2007	x			x		*C. bifurcatus* (gr. 1)
DU07-BA10	bathophilus	DP	2007	x	KF278321		x		*C. bifurcatus* (gr. 1)
DU10-BA1	bathophilus	DP	2010		KF278322	KF278437	x		*C. bifurcatus* (gr. 1)
DU10-BA2	bathophilus	DP	2010		KF278323	KF278440	x		*C. bifurcatus* (gr. 1)
OP07-BA1	bathophilus	OP	2007	x			x		*C. bifurcatus* (gr. 1)
OP07-BA2	bathophilus	OP	2007	x	KF278324		x		*C. bifurcatus* (gr. 1)
OP07-BA5	bathophilus	OP	2007		KF278325	KF278438	x		*C. bifurcatus* (gr. 1)
OP09-BA2	bathophilus	OP	2009		KF278326	KF278441	x		*C. bifurcatus* (gr. 1)
SJ07-BA4	bathophilus	SJ	2007	x	KF278352		x		*C. bifurcatus* (gr. 1)
SJ07-BA5	bathophilus	SJ	2007	x			x		*C. bifurcatus* (gr. 1)
KI06-BA1	bathophilus	KI	2006	x	KF278307		x		*C. bifurcatus* (gr. 2)
KI06-BA2	bathophilus	KI	2006	x			x		*C. bifurcatus* (gr. 2)
FA07-BA1	bathophilus	MC	2007	x			x		*C. bifurcatus* (gr. 2)
FA07-BA2	bathophilus	MC	2007	x	KF278310		x		*C. bifurcatus* (gr. 2)
FA07-BA3	bathophilus	MC	2007	x			x		*C. bifurcatus* (gr. 2)
FA07-BA4	bathophilus	MC	2007	x	KF278308		x		*C. bifurcatus* (gr. 2)
FA07-BA5	bathophilus	MC	2007	x			x		*C. bifurcatus* (gr. 2)
FA07-BA6	bathophilus	MC	2007	x			x		*C. bifurcatus* (gr. 2)
FA07-BA7	bathophilus	MC	2007	x			x		*C. bifurcatus* (gr. 2)
FA07-BA8	bathophilus	MC	2007	x	KF278311		x	x	*C. bifurcatus* (gr. 2)
FA07-BA9	bathophilus	MC	2007	x			x		*C. bifurcatus* (gr. 2)
FA07-BA10	bathophilus	MC	2007	x			x		*C. bifurcatus* (gr. 2)
SJ07-BA1	bathophilus	SJ	2007		KF278312		x		*C. bifurcatus* (gr. 2)
SJ07-BA2	bathophilus	SJ	2007		KF278313	KF278442	x		*C. bifurcatus* (gr. 2)
SJ07-BA3	bathophilus	SJ	2007		KF278314	KF278443	x		*C. bifurcatus* (gr. 2)

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
TI11-BA1	bathophilus	TI	2011		KF278309	KF278439	x	x	*C. bifurcatus* (gr. 2)
BO06-SA1	salinarius	BO	2006	x			x		*C. cucini*
BO06-SA2	salinarius	BO	2006	x			x		*C. cucini*
BO06-SA3	salinarius	BO	2006	x			x		*C. cucini*
BO06-SA4	salinarius	BO	2006	x			x		*C. cucini*
BO06-SA5	salinarius	BO	2006	x			x		*C. cucini*
BO06-SA6	salinarius	BO	2006				x	x	*C. cucini*
BO06-SA7	salinarius	BO	2006				x	x	*C. cucini*
CW07-SA1	salinarius	CL	2007	x	KF278288		x		*C. cucini*
CW07-SA2	salinarius	CL	2007	x	KF278289		x		*C. cucini*
CW07-SA3	salinarius	CL	2007	x			x		*C. cucini*
CW07-SA4	salinarius	CL	2007	x			x		*C. cucini*
CW07-SA5	salinarius	CL	2007	x			x		*C. cucini*
CW07-SA6	salinarius	CL	2007	x			x		*C. cucini*
CW07-SA7	salinarius	CL	2007	x			x		*C. cucini*
CW07-SA8	salinarius	CL	2007	x	KF278290		x	x	*C. cucini*
CW07-SA9	salinarius	CL	2007	x			x		*C. cucini*
CW07-SA10	salinarius	CL	2007	x			x		*C. cucini*
CW07-SA11	salinarius	CL	2007	x	KF278286		x		*C. cucini*
CW07-SA12	salinarius	CL	2007	x			x		*C. cucini*
CW07-SA13	salinarius	CL	2007	x			x		*C. cucini*
CW07-SA14	salinarius	CL	2007	x			x		*C. cucini*
CW07-SA15	salinarius	CL	2007	x			x		*C. cucini*
OP07-SA1	salinarius	OP	2007	x	KF278287		x		*C. cucini*
SJ06-SA1	salinarius	SJ	2006	x			x		*C. cucini*
SJ06-SA2	salinarius	SJ	2006	x			x		*C. cucini*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
SJ06-SA3	salinarius	SJ	2006	x			x		*C. cucini*
SJ06-SA4	salinarius	SJ	2006	x		KF278417	x		*C. cucini*
SJ06-SA5	salinarius	SJ	2006	x		KF278418	x		*C. cucini*
VA10-SA1	salinarius	VA	2010		KF278284	KF278419	x		*C. cucini*
VA10-SA2	salinarius	VA	2010		KF278285	KF278420	x	x	*C. cucini*
VA10-SA3	salinarius	VA	2010				x	x	*C. cucini*
AD10-BAFL1	fluviatilis	AD	2010		KF278298		x		*C. decorus*-group sp. 2
AD10-BAFL2	fluviatilis	AD	2010		KF278295		x	x	*C. decorus*-group sp. 2
DUF06-BAFL1	bathophilus	DF	2006	x			x		*C. decorus*-group sp. 2
DUF06-BAFL2	bathophilus	DF	2006	x			x		*C.* decorus-group sp. 2
DUF06-BAFL3	bathophilus	DF	2006	x			x		*C. decorus*-group sp. 2
DUF06-BAFL4	bathophilus	DF	2006	x	KF278293		x		*C. decorus*-group sp. 2
DUF06-BAFL5	bathophilus	DF	2006	x			x		*C. decorus*-group sp. 2
DP06-BAFL1	bathophilus	DP	2006	x			x		*C. decorus*-group sp. 2
DP06-BAFL3	bathophilus	DP	2006	x	KF278299		x		*C. decorus*-group sp. 2
DP06-BAFL4	bathophilus	DP	2006	x			x		*C. decorus*-group sp. 2
DP06-BAFL5	bathophilus	DP	2006	x			x		*C. decorus*-group sp. 2
FO06-BAFL1	bathophilus	FO	2006	x	KF278300		x		*C. decorus*-group sp. 2
OP09-BAFL1	bathophilus	OP	2009		KF278296		x		*C. decorus*-group sp. 2
OP09-BAFL2	bathophilus	OP	2009		KF278297		x		*C. decorus*-group sp. 2
SC10-BAFL1	bathophilus	SC	2010		KF278294		x		*C. decorus*-group sp. 2
SC10-BAFL3	bathophilus	SC	2010				x	x	*C. decorus*-group sp. 2
SI11-BAFL1	melanotus	SI	2011		KF278301		x		*C. decorus*-group sp. 2
KE10-PL1	plumosus	KE	2010		KF278333	KF278401	x		*C. dilutus*
KE10-PL3	plumosus	KE	2010		KF278337	KF278402	x		*C. dilutus*
KE10-PL7	plumosus	KE	2010		KF278338	KF278403	x		*C. dilutus*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
KE11-PL1	plumosus	KE	2011		KF278334	KF278404	x		*C. dilutus*
KE11-PL2	plumosus	KE	2011		KF278339	KF278405	x		*C. dilutus*
KE11-PL3	plumosus	KE	2011		KF278340	KF278406	x		*C. dilutus*
KE11-PL4	plumosus	KE	2011		KF278335	KF278407	x		*C. dilutus*
KE11-PL5	plumosus	KE	2011		KF278341	KF278408	x		*C. dilutus*
KE11-PL6	plumosus	KE	2011		KF278336	KF278409	x		*C. dilutus*
AL06-SRPL10	semireductus	DA	2006	x		KF278410	x	x	*C. entis*
DAS10-SRPL1	semireductus	DS	2010		KF278208	KF278411	x	x	*C. entis*
M-585	semireductus	MN	2007					x	*C. entis*
OP09-SRPL1	semireductus	OP	2009		KF278213	KF278412	x	x	*C. entis*
OP09-SRPL2	semireductus	OP	2009			KF278413	x	x	*C. entis*
PE10-SRPL1	semireductus	PE	2010		KF278212	KF278414	x	x	*C. entis*
PE10-SRPL2	semireductus	PE	2010			KF278415	x	x	*C. entis*
PE10-SRPL3	semireductus	PE	2010			KF278416	x	x	*C. entis*
AL06-SRPL3	plumosus	AL	2006	x					*C. entis* or *C. plumosus*
AL06-SRPL5	plumosus	AL	2006	x					*C. entis* or *C. plumosus*
AL06-SRPL8	plumosus	AL	2006	x					*C. entis* or *C. plumosus*
AL06-SRPL9	plumosus	AL	2006	x					*C. entis* or *C. plumosus*
AL06-SRPL6	plumosus	DA	2006	x	not published				*C. entis* or *C. plumosus*
AL06-SRPL7	plumosus	DA	2006	x	not published				*C. entis* or *C. plumosus*
DU07-SRPL2	semireductus to plumosus	DU	2007	x					*C. entis* or *C. plumosus*
DU07-SRPL6	semireductus to plumosus	DU	2007	x					*C. entis* or *C. plumosus*
DU07-SRPL7	semireductus to plumosus	DU	2007	x					*C. entis* or *C. plumosus*
DU07-SRPL8	semireductus to plumosus	DU	2007	x					*C. entis* or *C. plumosus*
DU07-SRPL9	semireductus to plumosus	DU	2007	x					*C. entis* or *C. plumosus*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
DU07-SRPL10	semireductus to plumosus	DU	2007	x					*C. entis* or *C. plumosus*
FO06-SRPL3	semireductus to plumosus	FO	2006	x					*C. entis* or *C. plumosus*
FO06-SRPL4	semireductus	FO	2006	x					*C. entis* or *C. plumosus*
FO06-SRPL5	semireductus	FO	2006	x					*C. entis* or *C. plumosus*
FO06-SRPL6	semireductus to plumosus	FO	2006	x					*C. entis* or *C. plumosus*
FO06-SRPL7	semireductus to plumosus	FO	2006	x					*C. entis* or *C. plumosus*
KI06-SRPL2	plumosus	KI	2006	x					*C. entis* or *C. plumosus*
KI06-SRPL3	plumosus	KI	2006	x					*C. entis* or *C. plumosus*
KI06-SRPL4	plumosus	KI	2006	x					*C. entis* or *C. plumosus*
KI06-SRPL5	plumosus	KI	2006	x					*C. entis* or *C. plumosus*
MA06-SRPL1	semireductus	MA	2006	x					*C. entis* or *C. plumosus*
MA07-SRPL2	semireductus to plumosus	MA	2007	x					*C. entis* or *C. plumosus*
MA07-SRPL3	semireductus	MA	2007	x					*C. entis* or *C. plumosus*
OP06-SRPL1	semireductus to plumosus	OP	2006	x					*C. entis* or *C. plumosus*
OS06-SRPL1	semireductus	OS	2006	x					*C. entis* or *C. plumosus*
PO07-PL1	plumosus	PO	2007	x			x		*C. frommeri*
PO07-PL3	plumosus	PO	2007	x			x		*C. frommeri*
PO07-PL5	plumosus	PO	2007	x			x		*C. frommeri*
PO07-PL6	plumosus	PO	2007	x	KF278235		x	x	*C. frommeri*
PO07-PL7	plumosus	PO	2007	x	KF278236		x		*C. frommeri*
PO07-PL8	plumosus	PO	2007	x			x		*C. frommeri*
PO07-PL9	plumosus	PO	2007	x	KF278237		x		*C. frommeri*
AR10-PL1	plumosus	AR	2010		KF278304	KF278430	x		*C. harpi*
AR10-PL2	plumosus	AR	2010		KF278306	KF278433	x		*C. harpi*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
AR10-PL3	plumosus	AR	2010		KF278302	KF278428	x		*C. harpi*
AR10-PL4	plumosus	AR	2010		KF278305	KF278431	x		*C. harpi*
AR10-PL5	plumosus	AR	2010		KF278303	KF278429	x		*C. harpi*
AR10-PL6	plumosus	AR	2010			KF278432	x		*C. harpi*
BD07-PL1	plumosus	BE	2007	x	KF278292		x		*C. maturus*
BD07-PL2	plumosus	BE	2007	x			x		*C. maturus*
BD07-PL3	plumosus	BE	2007	x			x		*C. maturus*
BD07-PL4	plumosus	BE	2007	x			x		*C. maturus*
BD07-PL5	plumosus	BE	2007	x			x		*C. maturus*
BD07-PL6	plumosus	BE	2007	x			x		*C. maturus*
BD07-PL7	plumosus	BE	2007	x			x		*C. maturus*
BD07-PL8	plumosus	BE	2007	x			x		*C. maturus*
BD07-PL9	plumosus	BE	2007	x	KF278291	KF278400	x		*C. maturus*
BD07-PL10	plumosus	BE	2007	x			x		*C. maturus*
MA09-TH1	thummi	MN	2009		KF278330		x	x	*C.* nr. *atroviridis* (sp. 2i)
MA09-TH2	thummi	MN	2009		KF278329		x	x	*C.* nr. *atroviridis* (sp. 2i)
MA09-TH3	thummi	MN	2009		KF278331		x		*C.* nr. *atroviridis* (sp. 2i)
MA09-TH4	thummi	MN	2009		KF278332		x		*C.* nr. *atroviridis* (sp. 2i)
OP09-TH1	thummi	OP	2009		KF278327		x	x	*C. ochreatus*
OP09-TH2	thummi	OP	2009		KF278328		x	x	*C. ochreatus*
AU10-SRPL1	plumosus	AU	2010		KF278214	KF278389	x	x	*C. plumosus*
AU10-SRPL2	semireductus to plumosus	AU	2010				x	x	*C. plumosus*
AU10-SRPL4	plumosus	AU	2010			KF278376	x	x	*C. plumosus*
AL06-SRPL1	plumosus	DA	2006	x		KF278384	x	x	*C. plumosus*
AL06-SRPL2	plumosus	DA	2006	x		KF278362	x	x	*C. plumosus*
AL06-SRPL4	plumosus	DA	2006	x			x	x	*C. plumosus*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
DU07-SRPL1	semireductus to plumosus	DP	2007	x			x	x	*C. plumosus*
DU10-SRPL1	semireductus to plumosus	DP	2010		KF278209	KF278363	x	x	*C. plumosus*
DU10-SRPL2	semireductus to plumosus	DP	2010			KF278377	x	x	*C. plumosus*
DU10-SRPL3	semireductus to plumosus	DP	2010			KF278364	x	x	*C. plumosus*
FO06-SRPL1	plumosus	FO	2006	x		KF278378	x	x	*C. plumosus*
FO06-SRPL2	plumosus	FO	2006	x		KF278380	x	x	*C. plumosus*
KE10-SRPL2	plumosus	KE	2010			KF278372	x	x	*C. plumosus*
KE10-SRPL3	plumosus	KE	2010			KF278365	x	x	*C. plumosus*
KE10-SRPL6	plumosus	KE	2010			KF278373	x	x	*C. plumosus*
KE10-SRPL8	plumosus	KE	2010			KF278379	x	x	*C. plumosus*
KI06-SRPL1	plumosus	KI	2006	x	KF278210		x	x	*C. plumosus*
MA07-SRPL1	semireductus	MN	2007	x	KF278216		x	x	*C. plumosus*
MA10-SRPL1	semireductus	MN	2010		KF278218	KF278385	x	x	*C. plumosus*
OS09-SRPL1	plumosus	OS	2009		KF278211	KF278366	x	x	*C. plumosus*
OS09-SRPL2	plumosus	OS	2009			KF278367	x	x	*C. plumosus*
OS09-SRPL3	plumosus	OS	2009			KF278375	x	x	*C. plumosus*
PE10-SRPL4	semireductus	PE	2010			KF278374	x	x	*C. plumosus*
PE10-SRPL5	semireductus to plumosus	PE	2010			KF278386	x	x	*C. plumosus*
PE10-SRPL6	plumosus	PE	2010		KF278217	KF278368	x	x	*C. plumosus*
PE10-SRPL7	semireductus to plumosus	PE	2010			KF278369	x	x	*C. plumosus*
PE10-SRPL8	plumosus	PE	2010			KF278381	x	x	*C. plumosus*
PE10-SRPL9	semireductus to plumosus	PE	2010			KF278382	x	x	*C. plumosus*
PE10-SRPL10	plumosus	PE	2010			KF278387	x	x	*C. plumosus*
PE10-SRPL11	semireductus to plumosus	PE	2010			KF278370	x	x	*C. plumosus*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
PE10-SRPL13	plumosus	PE	2010			KF278388	x	x	*C. plumosus*
RO10-SRPL1	plumosus	RO	2010		KF278215			x	*C. plumosus*
RO10-SRPL3	plumosus	RO	2010			KF278371	x	x	*C. plumosus*
KA07-TH1	thummi	KA	2007	x			x		*C.* sp. NAI
KA07-TH2	thummi	KA	2007	x			x		*C.* sp. NAI
KA07-TH3	thummi	KA	2007	x	KF278220		x		*C.* sp. NAI
KA07-TH5	thummi	KA	2007	x			x		*C.* sp. NAI
KA07-TH6	thummi	KA	2007	x		KF278390	x	x	*C.* sp. NAI
KA07-TH7	thummi	KA	2007	x	KF278219		x	x	*C.* sp. NAI
KA07-TH8	thummi	KA	2007	x			x	x	*C.* sp. NAI
KA07-TH9	thummi	KA	2007	x		KF278391	x		*C.* sp. NAI
KA07-TH10	thummi	KA	2007	x		KF278392	x		*C.* sp. NAI
SI07-SA1	salinarius	SI	2007	x			x		*C.* sp. NAII
SI07-SA2	salinarius	SI	2007	x	KF278270		x		*C.* sp. NAII
SI07-SA3	salinarius	SI	2007	x			x		*C.* sp. NAII
SI07-SA4	salinarius	SI	2007	x	KF278269		x		*C.* sp. NAII
AL06-SA1	salinarius	DA	2006	x	KF278276		x		*C.* sp. NAIII
AL06-SA2	salinarius	DA	2006	x			x		*C.* sp. NAIII
AL06-SA3	salinarius	DA	2006	x			x		*C.* sp. NAIII
AL06-SA4	salinarius	DA	2006	x			x		*C.* sp. NAIII
AL06-SA5	salinarius	DA	2006	x			x		*C.* sp. NAIII
AL06-SA6	salinarius	DA	2006	x			x		*C.* sp. NAIII
HA07-SA1	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA2	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA3	salinarius	HA	2007	x	KF278271		x		*C.* sp. NAIII
HA07-SA4	salinarius	HA	2007	x			x	x	*C.* sp. NAIII

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
HA07-SA5	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA6	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA7	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA8	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA9	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA10	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA11	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA12	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA13	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA14	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA15	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA16	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA17	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA18	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA19	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA07-SA20	salinarius	HA	2007	x			x		*C.* sp. NAIII
HA10-SA1	salinarius	HA	2010		KF278274	KF278423	x		*C.* sp. NAIII
FA07-SA1	salinarius	MC	2007	x	KF278281		x		*C.* sp. NAIII
FA07-SA2	salinarius	MC	2007	x			x		*C.* sp. NAIII
FA07-SA3	salinarius	MC	2007	x			x		*C.* sp. NAIII
FA07-SA4	salinarius	MC	2007	x			x	x	*C.* sp. NAIII
FA07-SA5	salinarius	MC	2007	x			x		*C.* sp. NAIII
FA07-SA6	salinarius	MC	2007	x			x		*C.* sp. NAIII
FA07-SA7	salinarius	MC	2007	x			x		*C.* sp. NAIII
FA07-SA8	salinarius	MC	2007	x			x		*C.* sp. NAIII
FA07-SA9	salinarius	MC	2007	x			x		*C.* sp. NAIII

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
RA10-SA1	salinarius	RA	2010		KF278277		x		*C.* sp. NAIII
RA10-SA3	salinarius	RA	2010		KF278272	KF278422	x		*C.* sp. NAIII
RA10-SA4	salinarius	RA	2010		KF278273	KF278424	x		*C.* sp. NAIII
RA10-SA5	salinarius	RA	2010		KF278283		x		*C.* sp. NAIII
RA10-SA6	salinarius	RA	2010		KF278278		x		*C.* sp. NAIII
RA10-SA7	salinarius	RA	2010		KF278279		x		*C.* sp. NAIII
RAM07-SA1	salinarius	RM	2007	x	KF278275		x		*C.* sp. NAIII
RAM07-SA2	salinarius	RM	2007	x			x		*C.* sp. NAIII
RAM07-SA3	salinarius	RM	2007	x			x		*C.* sp. NAIII
RAM07-SA4	salinarius	RM	2007	x	KF278282	KF278421	x		*C.* sp. NAIII
RAM07-SA5	salinarius	RM	2007	x	KF278280		x		*C.* sp. NAIII
RAM07-SA6	salinarius	RM	2007	x			x	x	*C.* sp. NAIII
RAM07-SA7	salinarius	RM	2007	x			x		*C.* sp. NAIII
RAM07-SA8	salinarius	RM	2007	x			x		*C.* sp. NAIII
RAM07-SA9	salinarius	RM	2007	x			x		*C.* sp. NAIII
RAM07-SA10	salinarius	RM	2007	x			x		*C.* sp. NAIII
CR10-PL1	plumosus	CR	2010		KF278257		x		*C. staegeri*
AL06-PL1	plumosus	DA	2006	x	KF278256		x		*C. staegeri*
AL06-PL2	plumosus	DA	2006	x			x		*C. staegeri*
AL06-PL3	plumosus	DA	2006	x			x		*C. staegeri*
AL06-PL4	plumosus	DA	2006	x			x		*C. staegeri*
AL06-PL5	plumosus	DA	2006	x			x		*C. staegeri*
DU06-PL2	plumosus	DP	2006	x			x		*C. staegeri*
DU07-PL3	plumosus	DP	2007	x			x		*C. staegeri*
DU07-PL4	plumosus	DP	2007	x	KF278258		x		*C. staegeri*
DU07-PL5	plumosus	DP	2007	x			x		*C. staegeri*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
DU10-PL1	plumosus	DP	2010		KF278259		x		*C. staegeri*
KA07-PL21	plumosus	KA	2007	x			x		*C. staegeri*
KI06-PL1	plumosus	KI	2006	x	KF278261		x		*C. staegeri*
FA07-PL1	plumosus	MC	2007	x	KF278260		x		*C. staegeri*
FA07-PL2	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL3	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL4	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL5	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL6	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL7	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL8	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL9	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL10	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL11	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL12	plumosus	MC	2007	x	KF278254		x		*C. staegeri*
FA07-PL13	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL14	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL15	plumosus	MC	2007	x			x		*C. staegeri*
FA07-PL16	plumosus	MC	2007	x			x		*C. staegeri*
OP09-PL1	plumosus	OP	2009		KF278262		x		*C. staegeri*
PO07-PL2	plumosus	PO	2007	x	KF278255		x	x	*C. staegeri*
PO07-PL4	plumosus	PO	2007	x			x		*C. staegeri*
SI07-PL1	plumosus	SI	2007	x			x		*C. staegeri*
SI07-PL2	plumosus	SI	2007	x			x		*C. staegeri*
SI07-PL3	plumosus	SI	2007	x			x		*C. staegeri*
SI07-PL4	plumosus	SI	2007	x			x		*C. staegeri*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
SI07-PL5	plumosus	SI	2007	x	KF278263		x		*C. staegeri*
SI07-PL6	plumosus	SI	2007	x			x		*C. staegeri*
SI07-PL7	plumosus	SI	2007	x			x		*C. staegeri*
SJ07-PL1	plumosus	SJ	2007		KF278264		x		*C. staegeri*
SJ07-PL2	plumosus	SJ	2007		KF278265		x		*C. staegeri*
SJ07-PL3	plumosus	SJ	2007		KF278268		x		*C. staegeri*
TI07-PL11	plumosus	TI	2007	x	KF278266		x		*C. staegeri*
TI11-PL1	plumosus	TI	2011		KF278267		x		*C. staegeri*
KA07-PL1	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL2	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL3	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL4	plumosus	KA	2007	x	KF278242		x		*C. 'tigris'*
KA07-PL5	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL6	plumosus	KA	2007	x	KF278243		x	x	*C. 'tigris'*
KA07-PL7	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL8	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL9	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL10	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL11	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL12	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL13	plumosus	KA	2007	x	KF278244		x		*C. 'tigris'*
KA07-PL14	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL15	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL16	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL17	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL18	plumosus	KA	2007	x			x		*C. 'tigris'*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
KA07-PL19	plumosus	KA	2007	x			x		*C. 'tigris'*
KA07-PL20	plumosus	KA	2007	x			x		*C. 'tigris'*
FA07-PL17	plumosus	MC	2007	x	KF278245		x		*C. 'tigris'*
FA07-PL18	plumosus	MC	2007	x			x		*C. 'tigris'*
FA07-PL19	plumosus	MC	2007	x			x		*C. 'tigris'*
FA07-PL20	plumosus	MC	2007	x	KF278253		x		*C. 'tigris'*
FA07-PL21	plumosus	MC	2007	x			x		*C. 'tigris'*
FA07-PL22	plumosus	MC	2007	x	KF278246		x		*C. 'tigris'*
FA07-PL23	plumosus	MC	2007	x			x		*C. 'tigris'*
FA07-PL24	plumosus	MC	2007	x			x		*C. 'tigris'*
FA07-PL25	plumosus	MC	2007	x			x		*C. 'tigris'*
FA07-PL26	plumosus	MC	2007	x			x		*C. 'tigris'*
FA07-PL27	plumosus	MC	2007		KF278247	KF278396	x		*C. 'tigris'*
OP06-PL1	plumosus	OP	2006		KF278238	KF278397	x		*C. 'tigris'*
OP07-PL1	plumosus	OP	2007	x			x		*C. 'tigris'*
OP07-PL2	plumosus	OP	2007	x			x		*C. 'tigris'*
OP07-PL3	plumosus	OP	2007	x			x		*C. 'tigris'*
OP07-PL4	plumosus	OP	2007	x			x		*C. 'tigris'*
OP07-PL5	plumosus	OP	2007	x			x		*C. 'tigris'*
OP07-PL6	plumosus	OP	2007	x	KF278239		x	x	*C. 'tigris'*
OP07-PL7	plumosus	OP	2007	x			x		*C. 'tigris'*
OP07-PL8	plumosus	OP	2007	x			x		*C. 'tigris'*
OP07-PL9	plumosus	OP	2007	x			x		*C. 'tigris'*
OP07-PL10	plumosus	OP	2007	x			x		*C. 'tigris'*
OP09-PL2	plumosus	OP	2009		KF278240	KF278398	x		*C. 'tigris'*
SI07-PL8	plumosus	SI	2007	x			x		*C. 'tigris'*

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TABLE S1. (Continued)

Voucher code	Larvae type	Locations	Performed analysis						Species
			Year	*cox1* PCR-RFLP	*cox1* sequencing (GenBank assession #)	*gb2β* sequencing (GenBank assession #)	Morphology	Cytology	
SI07-PL9	plumosus	SI	2007	x			x		*C. 'tigris'*
SI07-PL10	plumosus	SI	2007	x			x		*C. 'tigris'*
SI07-PL11	plumosus	SI	2007	x			x		*C. 'tigris'*
SI07-PL12	plumosus	SI	2007	x			x		*C. 'tigris'*
SI07-PL13	plumosus	SI	2007	x			x		*C. 'tigris'*
SI07-PL14	plumosus	SI	2007	x			x		*C. 'tigris'*
SI07-PL15	plumosus	SI	2007	x	KF278248		x		*C. 'tigris'*
SI07-PL16	plumosus	SI	2007	x			x		*C. 'tigris'*
SJ07-PL4	plumosus	SJ	2007	x	KF278249		x		*C. 'tigris'*
SJ07-PL5	plumosus	SJ	2007	x			x		*C. 'tigris'*
TI07-PL1	plumosus	TI	2007	x			x		*C. 'tigris'*
TI07-PL2	plumosus	TI	2007	x	KF278250		x		*C. 'tigris'*
TI07-PL3	plumosus	TI	2007	x			x		*C. 'tigris'*
TI07-PL4	plumosus	TI	2007	x	KF278241		x		*C. 'tigris'*
TI07-PL5	plumosus	TI	2007	x			x		*C. 'tigris'*
TI07-PL6	plumosus	TI	2007	x			x		*C. 'tigris'*
TI07-PL7	plumosus	TI	2007	x			x		*C. 'tigris'*
TI07-PL8	plumosus	TI	2007	x			x		*C. 'tigris'*
TI07-PL9	plumosus	TI	2007	x			x		*C. 'tigris'*
TI07-PL10	plumosus	TI	2007	x	KF278252		x		*C. 'tigris'*
TI11-PL2	plumosus	TI	2011		KF278251	KF278399	x		*C. 'tigris'*

TABLE S2. List of voucher *Chironomus* species sequenced.

Chironomus species	Location	Collector	Identified by	GenBank Accession #		Voucher number
				cox1	*gb2B*	
C. (*Chaetolabis*) nr. *atroviridis* (sp.2i)	White Lake, Three Mile Bay, ON, Canada	Don R. Oliver	Jon Martin	KF278342		Ch.sp2i15m
C. (*Chaetolabis*) nr. *atroviridis* (sp.2i)	White Lake, Three Mile Bay, ON, Canada	Don R. Oliver	Jon Martin	KF278360		Chaet.2i16m
C. (*Chaetolabis*) nr. *atroviridis* (sp.2i)	White Lake, Three Mile Bay, ON, Canada	Don R. Oliver	Jon Martin		KF278450	DRO.14.6 16M
C. (*Chaetolabis*) *ochreatus*	Little John Jr. Lake, WI, United States	Jon Martin	Jon Martin	KF278351		Ch.ochr13F
C. (*Chironomus*) *acidophilus*	Potters Marsh, Anchorage Co., AK, United States	Dave Wartenbee	Jon Martin	KF278358		UAK.1.14F or acidUAK1*
C. (*Chironomus*) *anthracinus*	Lake Esrom, Denmark	Henk Vallendduuk	Claus Lindegaard	KF278343		ES(DAN)95-BA3
C. (*Chironomus*) *bifurcatus*	Arboretum, Madison, Dane Co., WI, United States	Jon Martin	Jon Martin	KF278345		AAW4003*
C. (*Chironomus*) *bifurcatus*	Lake Pleasant, Franklin Co., MA, United States	Sean Werle	Jon Martin	KF278361		bifMa21
C. (*Chironomus*) *bifurcatus*	Arboretum, Madison, Dane Co., WI, United States	Jon Martin	Jon Martin	KF278353		bifMad7
C. (*Chironomus*) *calligraphus*	Gainsville, Alachua Co., FL, United States	Pauline O. Lawrence	Jon Martin	KF278357	KF278449	ABZ9507* or UFL.2.1 male4.1
C. (*Chironomus*) *dilutus*	Stevens Pond, Madison, Dane Co., WI, United States	Barry T.O. Lee	Jon Martin	KF278359		Stevens Pond_Madison WI (Eastern)
C. (*Chironomus*) *entis*	Saginaw Bay, Lake Michigan, MI, United States	Michael H. Winnell	Jon Martin	KF278355	KF278446	C.entisMI22 or UM1.3.1 22
C. (*Chironomus*) *entis*	Brewer Lake, Cass Co., ND, United States	Malcolm G. Butler	Jon Martin		KF278445	UND.2.1 2
C. (*Chironomus*) *harpi*	Bradleys Acid Pit, Jackson Co., IL, United States	Ken D. Yamamoto	Jon Martin	KF278346		AAJ4275*
C. (*Chironomus*) *plumosus*	Saginaw Bay, Lake Michigan, MI, United States	Michael H. Winnell	Jon Martin	KF278354	KF278444	C.plumMI21 or UM1.3.1 21
C. (*Chironomus*) *quinnitukqut*	Truro, Cape Cod, Barnstable Co., MA, United States	Jon Martin	Jon Martin	KF278347		AAB7030*
C. sp. g	Lake Bat, Algonquin Park, ON, Canada	Jon Martin	Jon Martin	KF278348		C.spgBatLk
C. sp. h	Lake Bat, Algonquin Park, ON, Canada	Jon Martin	Jon Martin	KF278349		C.sphBatLk
C. sp. u	Calgary, AL, Canada	Jon Martin	Jon Martin		KF278447	CAL.2.4 egg mass #3, 3.2f
C. (*Chironomus*) *staegeri*	Lake Pleasant, Franklin Co., MA, United States	Jon Martin	Jon Martin	KF278356		AAW3999*
C. (*Chironomus*) '*tigris*'	Turtle Lake, Becker Co., MN, United States	Malcolm G. Butler	Jon Martin	KF278350		C.tigris_TurtleLk_MN_USA
C. (*Chironomus*) *anthracinus*-gr.	Marion Lake, Garibaldi Prov.Pk., BC, Canada	Andrew L. Hamilton	Andrew L. Hamilton	KF278344		CBC.1.1 14f(1)

*Published in BOLD (BIN #)

TABLE S3. Mean and range of intraspecific sequence divergences of the *cox1* and *gb2β* genes for collected and reference *Chironomus* species.

Chironomus species	*cox1*			*gb2β*		
	No. of specimens	K2P divergence (%)		No. of specimens	K2P divergence (%)	
		Mean	Range		Mean	Range
C. anthracinus	16	0.06	0.00–0.15	6	0.34	0.00–0.86
C. bifurcatus	25	1.24	0.00–2.60	10	0.29	0.00–0.97
C. cucini	7	0.21	0.00–0.48	4	0.21	0.00–0.32
C. decorus–group sp. 2	10	0.26	0.00–0.61	0		
C. dilutus	11	1.34	0.00–3.26	11	0.00	0.00–0.00
C. entis	5	1.30	0.31–2.16	9	0.25	0.00–1.10
C. frommeri	3	0.41	0.00–0.61	0		
C. harpi	6	0.22	0.00–0.66	6	0.00	0.00–0.00
C. maturus	3	0.21	0.15–0.31	1		
C. nr. *atroviridis* (sp. 2i)	6	1.33	0.00–2.65	0		
C. ochreatus	3	0.10	0.00–0.15	0		
C. plumosus	9	0.93	0.00–1.54	29	0.64	0.00–2.23
C. sp. NAI	3	0.82	0.15–1.23	3	0.00	0.00–0.00
C. sp. NAII	2	0.77		0		
C. sp. NAIII	13	0.35	0.00–0.77	4	0.00	0.00–0.00
C. staegeri	16	0.04	0.00–0.31	0		
C. 'tigris'	17	0.15	0.00–0.61	4	0.00	0.00–0.00

TABLE S4. Bases that differ between *cox1* sequences of *C.* sp. NAI and *C. anthracinus*. Refer to Fig. 8 for sequence label.

Species	Sequences	Base position																					
		7	1	1	2	2	2	3	3	4	4	4	4	4	4	5	5	5	5	5	5	6	6
		6	1	2	6	3	0	4	8	4	9	5	3	5	8	0	0	8	6	1	2	8	2	6
C. sp. NAI	thummi (n=1 / KA / KF2782219)	A	C	G	C	C	T	A	C	G	A	A	C	G	T	C	A	C	A	T	T	C	T
C. sp. NAI	thummi (n=1 / KA / KF278220)	A	C	G	C	C	T	A	C	G	A	A	C	G	T	C	A	C	A	T	T	C	T
C. sp. NAI	*C. anthracinus*-group (KF278344)	A	C	G	C	C	T	A	C	G	A	A	C	G	T	C	A	C	A	T	T	C	T
C. anthracinus	thummi_(n=4 / HA, OS, PI / KF278221-KF278224)	G	T	A	T	T	C	G	T	A	G	G	T	A	C	T	G	T	G	C	C	T	C
C. anthracinus	thummi_(n=10 /AR, OS, PI, RA, RM, SI / KF278225-KF278234)	G	T	A	T	T	C	G	T	A	G	G	T	A	C	T	G	T	G	C	C	T	C
C. anthracinus	*C. anthracinus* (KF278343)	G	T	A	T	T	C	G	T	A	G	G	T	A	C	T	G	T	G	C	C	T	C

TABLE S5. Bases that differ between *cox1* sequences of *C. bifurcatus* groups 1 and 2. Refer to Fig. 8 for sequence label.

Species	Sequences	Base position									
		1	2	3	3	3	4	4	4	5	6
		9	7	3	4	8	3	7	9	1	2
		9	1	7	3	2	3	8	9	7	2
C. bifurcatus (gr. 1)	bathophilus (n=1 / DA / KF278315)	C	C	T	T	A	G	T	C	C	A
C. bifurcatus (gr. 1)	bathophilus (n=11 / AD, AR, DP, OP / KF278316-KF278326)	C	C	T	T	A	G	T	C	C	A
C. bifurcatus (gr. 1)	*C. bifurcatus* (KF278361)	C	C	T	T	A	G	T	A	C	A
C. bifurcatus (gr. 1)	bathophilus (n=1 / SJ / KF278352)	C	C	T	T	A	G	T	C	C	A
C. bifurcatus (gr. 2)	*C. bifurcatus* (KF278353)	T	A	A	C	T	A	A	T	T	C
C. bifurcatus (gr. 2)	*C. bifurcatus* (KF278345)	T	A	A	C	T	A	A	T	T	C
C. bifurcatus (gr. 2)	bathophilus (n=1 / KI / KF278307)	T	G	A	C	T	A	A	T	T	C
C. bifurcatus (gr. 2)	bathophilus (n=3 / SJ / KF278312-KF278314)	T	G	A	C	T	A	A	T	T	C
C. bifurcatus (gr. 2)	bathophilus (n=2 / MC / KF278310-KF278311)	T	G	A	C	T	A	A	T	T	T
C. bifurcatus (gr. 2)	bathophilus (n=2 / MC, TI / KF278308-KF278309)	T	G	A	C	T	A	A	T	T	T